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EFFECTS OF AMMONIA ON THE DISEASE SUSCEPTIBILITY OF FISH

Ana Filipa dos Santos Gonçalves

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Orientador – Professor Doutor João Coimbra
Categoria – Professor Catedrático
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Co-orientador – Doutor Jonathan Wilson
Categoria – Investigador Auxiliar
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental da Universidade do Porto

Co-orientador – Professor Mathilakath M. Vijayan
Categoria – Professor
Afiliação – University of Waterloo, Canada

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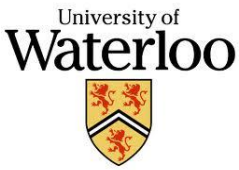

Dissertation organization

The present PhD thesis is organized in chapters, starting with General Introduction and finishing with General Discussion. Chapters 2, 3 and 4 correspond to the experimental work and are structured as papers. Each chapter will give origin to a separate paper and, within this dissertation, they are free standing, which may lead to a certain degree of repetition throughout the thesis. Tables and figures have sequential numbering from one chapter to the next. The bibliography is consolidated at the end of the thesis.

Abstract

Ammonia is an unusual toxicant in that it is not only a common environmental pollutant but is also an endogenous by-product of amino acid catabolism within the organism. In aquaculture, ammonia toxicity can be problematic when fish are held at high densities with insufficient water flow, or when using recirculating water systems, which may lead to elevated ammonia levels. Fish can also be exposed to elevated ammonia levels in the environment as a result of an input of biological wastes, runoff from fertilizer application for agriculture activities and atmospheric deposition. High environmental ammonia (HEA) is toxic to fish causing problems at multiple levels of organization, and has been associated with increased vulnerability to different parasitic, viral and bacterial diseases in fishes, although the mechanism(s) is not well understood.

The aim of this Ph.D. thesis was to provide new insights into the complex relationship between HEA levels and increased disease susceptibility using the zebrafish, *Danio rerio*, as the animal model. The hypotheses addressed were that: i) HEA acts by impairing the acute phase response (APR), and ii) cortisol, the principal corticosteriod in teleosts, mediates that immunosuppression.

The results revealed, for the first time, that HEA exposure impaired lipopolysaccharide (LPS) and bacterial (*Edwarsiella tarda*) -induction of various acute phase proteins (APPs) in zebrafish. And both acute and chronic exposure to HEA concentrations also significantly elevated whole-body cortisol levels compared with control fish. Significantly, HEA was found to decrease survival during bacterial challenge with *E. tarda.* In addition, it was demonstrated that exogenous exposure to the stress hormone cortisol, and the elevation of cortisol levels in response to HEA may be playing a key role in the downregulation of important innate immune genes and upregulation of suppressors of cytokine signaling (SOCS) genes. Mifepristone, a glucocorticoid receptor (GR) antagonist, blocked the HEA-cortisol effect through a GR mediated pathway. Given the similar direct effects of HEA and cortisol, it is possible to infer that the ammonia exposure effect on immunosuppression was mediated by cortisol.

The upregulation of *socs* gene expression by HEA/cortisol may be an adaptive response that limits infection-induced inflammatory responses and the associated metabolic costs by restricting activation of cytokine signalling, and diverting energy resources away from the growth promoting action of growth hormone during stress in fish. This would reallocate energy substrates away from anabolic processes, thereby allowing ATP usage for other processes, including production of glucose, a key fuel for coping with the increase energy demand associated with stress.

There are various studies showing that LPS induces *socs* expression. However, *socs* induction is not always observed with LPS challenge and there are data showing no changes or even a *socs3* down regulation. In this study, LPS challenge supressed *socs* transcript levels. To this point, the precise role of Socs proteins in LPS responses clearly remains enigmatic.

To summarize, the work presented in this thesis demonstrates that ammonia suppresses the immune response, at least in part, through an increase in cortisol levels in zebrafish, and ultimately increases the disease susceptibility associated with HEA levels in zebrafish. This is of particular relevance for maintenance of healthy fish in aquaculture, and for the monitoring of environmental health in wild fish populations faced with pollution.

Resumo

A amónia é um agente tóxico invulgar na medida em que é um poluente ambiental comum e é também um subproduto do catabolismo dos aminoácidos nos organismos. Em aquacultura é problemática quando os peixes são mantidos em altas densidades, com fluxo mínimo de água, ou quando se utilizam sistemas de recirculação de água, o que pode levar a níveis elevados de amónia. Os peixes também estão expostos a amónia no meio ambiente como resultado de resíduos biológicos, escoamento da aplicação de fertilizantes na agricultura e deposição atmosférica. Amónia ambiental elevada é tóxica para peixes e causa problemas a diferentes níveis: organismo, órgão e célula; e tem sido associada ao aumento da vulnerabilidade a diferentes doenças causadas por parasitas, vírus e bactérias em peixes, embora o mecanismo envolvido não seja bem compreendido.

O objetivo desta tese de doutoramento foi compreender melhor a relação complexa entre níveis elevados de amónia ambiental e a susceptibilidade aumentada a doença, usando o peixe-zebra, *Danio rerio*, como animal modelo. As hipóteses estudadas foram que a amónia age através da inibição da resposta de fase aguda; e também, que o cortisol, o corticosteroide principal em peixes teleósteos, é o mediador dessa imunossupressão.

Os resultados revelaram, pela primeira vez, que a amónia inibe a indução de várias proteínas de fase aguda, por lipopolissacarídeo bacteriano e por bactéria (*Edwardsiella tarda*) em peixes-zebra. Exposição aguda ou crónica a concentrações elevadas de amónia elevaram significativamente os níveis de cortisol em comparação com os peixes controle. Além disso, a amónia ambiental diminuiu a sobrevivência durante infeção bacteriana com *Edwarsiella tarda*. Também foi demonstrado que a hormona do stress, cortisol, e os elevados níveis de cortisol em resposta à exposição a amónia podem ser a chave para um aumento da indução de *socs*, e, uma diminuição da indução de genes importantes da resposta de fase aguda. Mifepristona, um potente antagonista do recetor de glucocorticoides, atenuou a ação do cortisol e os seus efeitos através de uma via mediada pelo recetor de glucocorticoides, nos grupos expostos a amónia. Devido aos efeitos semelhantes da amónia/cortisol, é possível deduzir que o efeito da amónia na imunossupressão foi mediado

pelo cortisol.

O aumento da indução da expressão dos genes *socs* por amónia/cortisol pode ser uma resposta adaptativa que limita a resposta inflamatória imunitária e os custos metabólicos associados, ao limitar a ativação da sinalização de citocinas, e também desviando recursos energéticos do crescimento, durante situações de stress em peixes. Isto permite realocar substratos energéticos dos processos anabólicos, permitindo assim o uso de ATP para outros processos, tais como a produção de glucose, para lidar com o aumento da demanda de energia associada ao stress. Existem vários estudos que demonstram que LPS leva à indução de expressão de *socs*. Contudo, a indução de *socs* não é sempre observada com LPS, há dados que mostram não existirem diferenças na expressão ou até diminuição da expressão de *socs3.* Neste estudo, LPS suprimiu a expressão de *socs*. Neste momento, o papel preciso das proteínas SOCS nas respostas ao LPS claramente permanece enigmático.

Resumindo e concluindo, o trabalho apresentado nesta tese mostra que elevados níveis de amónia ambiental atuam, pelo menos em parte, através do aumento nos níveis de cortisol em peixe-zebra, o que leva uma imunossupressão, e, finalmente, aumenta a susceptibilidade à doença associada com amónia em peixes. Isto é de particular relevância para a manutenção de peixes em aquacultura e à monitorização ambiental nas populações de peixes selvagens sujeitas a poluição.

List of Abbreviations

3β-HSD - 3β-hydroxysteroid dehydrogenase

ACTH - Adrenocorticotropic hormone

APP - Acute Phase Protein

APR - Acute phase response

ATP - Adenosine-5'-triphosphate

C3b / *c3b* - Complement Component

CT - Cycle threshold

EF1α / *ef1α* - Elongation factor-1 alpha

CFU - Colony-forming unit

CRH - corticosteroid releasing hormone

CRP / *crp* - C-reactive protein

GH – Growth hormone

GR / *gr* – Glucocorticoid receptor

GC - Glucocorticoids

HAMP / *hamp* – Hepcidin

HEA - High environmental ammonia

Hp / *hp* – Haptoglobin

HPI - hypothalamus-pituitary-interrenal

Iap / *iap* - Intestinal alkaline phosphatase

IL1β / *il1β* - Interleukin 1 beta

IL10 / *il10* - Interleukin 10

LC50 - Lethal concentration, 50%

LECT2 / *lect2* - Leukocyte cell-derived chemotaxin-2

LITAF / *litaf* - Lipopolysaccharide-induced TNF factor

LPS – Lipopolysaccharide

MS-222 - Tricaine methanesulfonate

NADH – Reduced nicotinanide adenine dinucleotide

NH_3 – Unionized ammonia

NH_4^+ - Ammonium ion

NMDA - *N*-methyl-D-aspartate

PBS - Phosphate-buffered saline

PCA - Perchloric acid

RT-PCR – Reverse transcriptase-polymerase chain reaction

SAA / *saa* - Serum amyloid A

SEM – Standard error of the mean

SOCS /*socs* - Suppressor of cytokine signaling

TAN - Total ammonia nitrogen

TBE - Tris-borate-EDTA

TLR / *tlr* - Toll-like receptor

TNFα / *tnfα* - Tumor necrosis factor alpha

TRIS - tris(hydroxymethyl)aminomethane

TSB - Tryptic soy broth

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CHAPTER 1

GENERAL INTRODUCTION

There are various studies showing that exposure to high environmental ammonia levels increase susceptibility disease in fish; however, the mechanism of action of ammonia remains largely unknown. This PhD thesis will try to provide new insights into the complex relationship between ammonia, the innate immune system and the increase disease vulnerability in zebrafish, and its link with the stress hormone cortisol. These factors will be discussed in more detail in the following sections.

PART I – AMMONIA

Ammonia is an unusual toxicant in that it is environmentally relevant and it is also produced naturally as a metabolic waste of protein catabolism within the organism. Proteins are hydrolyzed into amino acids and their deamination results in ammonia. The liver is the major organ for ammonia formation (Pequin and Serfaty, 1963), although also occurring in other organs like kidney and intestine (Walton and Cowey, 1977). Ammonia is also released into the environment through the application of industrial fertilizers and input of biological wastes, industrial emissions, decomposition of vegetation and animals, excretion by aquatic animals and volcanic activity (Ip et al., 2004; USEPA, 1999, Randall and Tsui, 2002). A major concern regarding ammonia toxicity is in aquatic systems, in regions of high human habitation and/or large numbers of farm animals, because urban and agricultural runoff and most biological waste are released into rivers and oceans (Randall and Tsui, 2002). In aquaculture, ammonia toxicity is problematic when using recirculating water systems, or during rearing stages in which fishes are held at high densities with minimal water flow, which may lead to high levels of ammonia.

Ammonia has been shown to have significant adverse neurological and physiological effects in vertebrates. At toxic internal levels, ammonia is detrimental to central nervous system processes, oxidative metabolism, and it may also impair oxygen delivery (Randall and Tsui, 2002; Wilkie, 1997). In aqueous solution ammonia exists as either unionized ammonia (NH_3) or ammonium ion (NH_4^+) in an equilibrium that is largely pH dependent ($NH_3 + H^+$

<-> NH_4^+, pK 9.2). Ammonia causes convulsions, coma and death, probably because elevated NH_4^+ displaces K^+ and depolarizes neurons, causing activation of NMDA (*N*-methyl-D-aspartate) type glutamate receptor, which leads to an influx of excessive Ca^{2+} and subsequent cell death in the central nervous system (Randall and Tsui, 2002). Exposure to high environmental ammonia (HEA) also causes gill hyperplasia, anemia, hypercortisolemia and ionoregulatory problems (USEPA, 1999; Ip et al., 2004; Wilkie, 2002).

Fish are generally ammonotelic and rely mainly on passive diffusion down the concentration gradient between the body and water for elimination. If the concentration in surrounding water exceeds that of blood (reverse diffusion gradient), ammonia becomes difficult to remove, and reducing production and/or conversion to nontoxic forms becomes a priority to avoid accumulation to toxic levels. Higher vertebrates make use of uric acid and urea (Wright et al., 1995). However, fish can tolerate relatively higher internal levels of ammonia than mammals, but ammonia accumulation still leads to cell and animal death through its impairment of energy metabolism, by interfering in the Krebs cycle and, more importantly, through its interference with cellular ion and acid-base homeostasis (Ip et al., 2004).

Ammonia is also generally accepted to increase susceptibility to different parasitic, bacterial and viral fish diseases (Carballo et al., 1995; Carballo and Munõz, 1991; Evans et al., 2005; Hanson and Grizzle, 1985; Liu, 2004). For example, juvenile Chinook salmon (*Oncorhynchus tshawytscha*) previously exposed to 0.12–0.49mM total ammonia nitrogen (TAN)[1] levels were more susceptible to *Vibrio anguillarum* challenge (Ackerman et al., 2006). Also, infection caused by *Tetrahymena sp.* was significantly higher when guppies (*Poecilia reticulata*) were exposed to 0.11– 0.32mM TAN (Pimenta-Leibowitz et al., 2005). In addition, Channel catfish, *Ictalurus punctatus*, injected with *Aeromonas hydrophila* and exposed to 0.11mM TAN[1] had significantly higher total bacterial counts than controls (Walters and Plumb, 1980). However, the mechanism of action of ammonia remains largely unknown. Detrimental effects are not always observed with ammonia exposure. Lease and collaborators (2003) showed that none of the more traditional toxicity endpoints (growth, whole-body ion content and swimming performance) were significantly affected by a range of TAN up to 1.71mM[1] in Lost River suckers. Also, TAN concentrations of 0.24mM[1] did not affect the antibody response to *Aeromonas salmonicida* in sunshine bass (Hrubec et al., 1996). Morris and collaborators

(2006) even demonstrated that survival of *Deltistes luxatus* exposed to *Flavobacterium columnare* increased significantly as unionized ammonia concentrations increased from 0 to 0.72mM TAN[1]. It is known that elevated ammonia concentrations cause gill damage in various fish species (Smart, 1976; Lang et al., 1987) but it remains unclear if this damage causes an increased susceptibility to infection.

PART II – INNATE IMMUNE SYSTEM

The immune system protects organisms from infection with layered defenses of increasing specificity. In simple terms, physical barriers prevent pathogens such as bacteria and viruses from entering the organism. If a pathogen breaches these barriers, the innate immune system provides an immediate, but non-specific response. Innate immune systems are found in all plants and animals (Litman et al., 2005). If pathogens successfully evade the innate response, vertebrates possess a second layer of protection, the adaptive immune system, which is activated by the innate response. Here, the immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated, in the form of an immunological memory, and allows the adaptive immune system to mount faster and stronger attacks each time this pathogen is encountered (Mayer, 2006).

The innate immune system is a universal and ancient form of host defense against infection (Janeway and Medzhitov, 2002). The elements of the innate immune system include anatomical barriers, secretory molecules and cellular components. The anatomical barriers are very effective in preventing colonization of tissues by microorganisms. However, when there is damage to tissues the anatomical barriers are breached and infection may occur. Once infectious agents have penetrated tissues, another innate defense mechanism comes into play, namely acute inflammation Mayer, 2006).

The acute phase response (APR) is the immediate set of host inflammatory reactions that counteract challenges such as tissue injury, infection and trauma; and it involves metabolic changes in several organ systems (Bayne et al., 2001; Nicolas et al., 1987; Uhlar and Whitehead, 1999). APR has been extensively studied in human and mouse.

One clear indication of the APR is the remarkable increase in the concentrations of many plasma proteins that are synthesized in hepatocytes, known as acute phase proteins (APPs; Gabay and Kushner, 1999; Uhlar and Whitehead, 1999). An APP has been defined as one whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 25 percent during inflammatory disorders. Some APPs were

isolated from fish (Bayne and Gerwick, 2001). The APPs function in a variety of defense-related activities, such as limiting the dispersion and killing pathogens, activating the complement system, neutralizing enzymes, scavenging free hemoglobin and radicals, repair of tissue damage, inactivation of proteases and restoration of the healthy (homeostatic) state that prevailed before the stimulus (Bayne and Gerwick, 2001; Gabay and Kushner, 1999; Gruys et al., 2005). In mammals, induction of acute phase proteins (APPs) is mediated by Toll-like receptors (TLRs), pro-inflammatory cytokines and CCAAT/enhancer-binding proteins (or C/EBPs). Upon infection, TLRs can recognize the microbial components thus sensing the invasion of pathogens (Akira and Takeda, 2004). Stimulation of these receptors finally results in the production of pro-inflammatory cytokines, such as interleukin 1, interleukin 6, and TNFα, which are important inducers of APPs (Cohen, 2002). These cytokines in turn induce the production of some important transcription factors of many APP genes (Poli, 1998).

Cytokines are an integral component of the adaptive and innate immune responses. Signal transduction via cytokine receptors is regulated by several mechanisms that control initiation, magnitude and duration of signaling pathways. Cytokine-induced suppressor of cytokine signaling (SOCS) family acts as feedback inhibitors of cytokine receptor signaling by inhibiting a variety of signal transduction pathways (Alexander and Hilton, 2004; Jin et al., 2007).

In this PhD thesis, I stimulated the innate immunity response in zebrafish by injection with *Escherichia coli* lipopolysaccharides (LPS). LPS are also termed endotoxins and are considered to be a major virulence factor, being responsible for lethal effects and clinical manifestations of diseases in humans and animals (Swain et al., 2008). It has been shown in many studies, that LPS can induce the innate immune response in fish (Huttenhuis et al., 2006; Watzke et al., 2007). LPS is a cell wall component found in most Gram-negative bacteria. However, some Gram-positive bacteria, such as *Listeria monocytogenes*, also possess endotoxin-like activity (Wexler and Oppenheim, 1979). Higher vertebrates are extremely sensitive to endotoxins, even at low doses, but lower vertebrates such as amphibians and fish are found to be resistant to endotoxic shock. Fish like *Oncorhynchus kisutch* and *Oncorhynchus mykiss* are reported to be resistant to *Escherichia coli* and *Aeromonas salmonicida* endotoxins in doses up to 80 mg/kg body weight (Wedemeyer et al., 1968).

Also, I challenged zebrafish using the fish pathogen *Edwarsiella tarda*, a

member of the Enterobacteriacae family, which was chosen because it grows at temperatures suitable for zebrafish maintenance and it has been established as a significant pathogen in a variety of fish species. The species most commonly associated with infection with this bacterium are *Ictalurus punctatus* (channel catfish), *Anguilla japonica* (Japanese eel) and *Paralichthys olivaceus* (Japanese flounder; Pressley et al., 2005). *Edwarsiella tarda* causes Edwardsiellosis, a generalized septicemia in a broad range of hosts, including humans, birds, reptiles, amphibians and aquatic mammals, in addition to fishes (Ling et al., 2001). Natural infection of fish is through waterborne contact with *E. tarda*. Ling and colleagues demonstrated that the gastrointestinal tract, gills and body surface of fish were the sites of entry by histological and infection kinetics studies (Ling et al., 2001). In addition, *E. tarda* was chosen for this study because Pressley and colleagues already showed that adult zebrafish were susceptible to challenge by intraperitoneal injection and immersion and determined that *E. tarda* infection induced a typical acute inflammatory response (Pressley et al., 2005).

PART III – CORTISOL

Cortisol is a key modulator of physiological processes; including the stress response, metabolism, growth and the immune response. It is the principal corticosteriod in teleost fishes and its plasma concentrations rise dramatically during stress (Mommsen et al., 1999). In teleosts, cortisol is released from the interrenal tissue (analogous to the adrenal cortex), distributed in the head kidney region; and it is elevated during stress following activation of the hypothalamus-pituitary-interrenal (HPI) axis (Aluru and Vijayan, 2009).

Neuroendocrine response to stress after perception by the sensors of the nervous system involves the immediate secretion of corticosteroid releasing hormone (CRH) by the preoptic nucleus of the hypothalamus. The stimulated CRH receptors in the corticotropic cells of the pituitary gland induce release of adrenocorticotropic hormone (ACTH) into the circulation that subsequently stimulates release of cortisol by the head kidney interrenal cells. ACTH as well as melanocyte-stimulating hormone are derived from cleavage of the pro-opiomelanocortin gene product (Tort, 2011).

Glucocorticoids produce their effect on responsive cells by acting through the glucocorticoid receptor (GR), which regulates the transcription of target genes. In the absence of a ligand, the GR is a transcriptionally inactive cytoplasmic protein that is part of a chaperone-containing multiprotein complex, which maintains a high affinity for the ligand. Upon hormone binding, the GR translocates to the nucleus, where it acts as a transcription factor. The GR binds DNA at glucocorticoid response elements in the promoter regions of corticosteroid-responsive genes, inducing transcription (Kassel and Herrlich, 2007; Schoneveld, 2004).

The biosynthesis of cortisol in fish is similar to that in mammals and involves the microsomal enzymatic pathways, including 21-hydroxylation (P450c21), 17α- hydroxylation (P450c17), and 3β-hydroxy steroid dehydrogenation (3β-HSD, Figure 1). In addition, fish possess the mitochondrial inner membrane monooxygenase enzymes, such as the cholesterol side-chain cleavage enzyme (cytochrome P450scc, desmolase) and the 11β-hydroxylase that catalyses the 11β- hydroxylation of deoxycortisol/deoxycorticosterone

(cytochrome P450c11) (Figure 1.1; Mommsen et al., 1999).

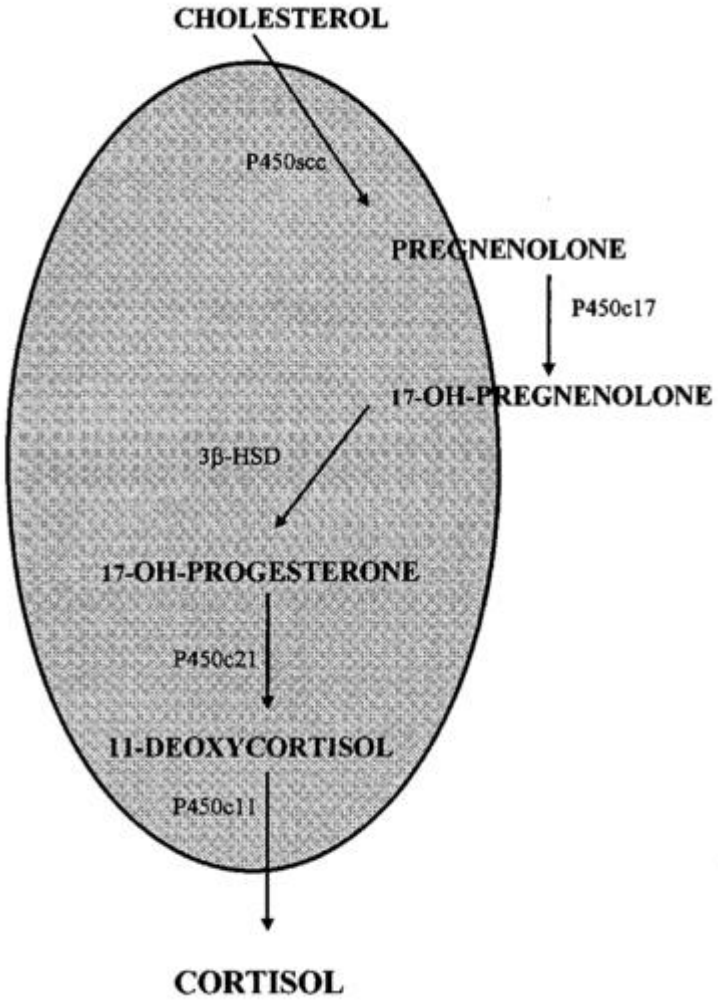

Fig. 1.1: Biosynthesis of cortisol in teleost fishes. The shaded area represents the mitochondrial compartment, the non-shaded area represents the cytosolic compartment. Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450s, various forms of cytochrome P450 (Mommsen et al., 1999).

Glucocorticoids (GC) appear to be potent immunosuppressive agents in vertebrates. And in fish, immune suppression has been observed when high levels of cortisol are secreted: cortisol and immune indicators change concurrently after repeated sampling stress in trout causing a reduction of immune competence (Sunyer and Tort, 1995). Severe immune suppression in cold-shock affected sea bream is correlated with high levels of cortisol (Tort et al., 1998). High density stress induces reduced levels of complement together with high cortisol in sea bream (Montero et al., 1999; MacKenzie et al., 2006). Cortisol inhibits inflammatory cytokine expression *in vitro* (Saeij et al., 2003).

Cortisol and lipopolysaccharide (LPS) synergistically stimulate expression of interleukin 1 (IL-1) mRNA in head kidney phagocytes (Engelsma et al., 2003). At the transcript level when macrophages are treated with LPS and cortisol, expression of genes involved in the inflammatory process are mostly down regulated and some of the basic cellular activities, like energetic metabolism, and protein biosynthesis are recovered (MacKenzie et al., 2006). When cortisol and LPS are added to macrophages, cytokine induction is down regulated by cortisol (Castillo et al., 2008). Similarly, LPS-induced expression of TNF decreases by adding cortisol and this effect is reverted by addition of mifepristone (Tort, 2011). Mifepristone (RU 486) is an anti-glucocorticosteroid that exerts its effects at the receptor and possibly the post-receptor level of steroid action. This antihormone has been used in a number of fish studies to block the effects of exogenously added cortisol and to confirm conclusively that cortisol was indeed the active principle behind the noted effects (Mommsen et al., 1999).

PART IV - THE EXPERIMENTAL ANIMAL MODEL

The zebrafish, *Danio rerio* (Figure 1.2), was the species used in the thesis. It is a tropical, freshwater fish, whose natural habitat is found in India, Pakistan and Bhutan. The zebrafish is a teleost fish and belongs, together with the carp and the goldfish, to the family of Cyprinidae (van der Sar et al., 2004). It is a popular aquarium fish and is one of the most widely used vertebrate models (Phelps and Neely, 2005; Van der Sar et al., 2004).

Fig. 1.2: The zebrafish, *Danio rerio*.

Zebrafish have a fully developed immune system with both innate and adaptive immune responses, allowing studies that involve both systems (Trede et al., 2004) and is an emerging model species for the study of fish as well as human diseases (Kari et al., 2007). In this regard, several research groups have identified pathogens that can infect and cause disease in zebrafish (Pressley et al., 2005; Neely et al., 2002; Davis et al., 2002; Prouty et al., 2003).

The zebrafish has many advantages when compared to other animal models. Among animals with a fully developed adaptive and innate immune system, the zebrafish is one of the smallest (≤5 cm), allowing large numbers of fish to be housed in a relatively small space. They are prolific, with a given pair able to produce 200–300 new progeny each week (Meeker and Trede, 2008).

Under natural conditions, pathogens are thought to infect zebrafish through the gastrointestinal tract, the gills or through the (damaged) fish surface (O'Toole et al.,2004). A gnotobiotic zebrafish model has been established to dissect the molecular interactions between host and microbes in the digestive tract (Rawls et al., 2004). Experimental infection of zebrafish can also be achieved by incubation in media containing the bacteria. This method has been used both for embryos (Davis et al., 2002) and for adult fish (Neely et al., 2002). However, experimental infections are usually introduced by intraperitoneal injection in anaesthetized fish. This enables accurate adjustment of the infection dose.

PART V - THESIS AIMS

The proposed goals of this PhD thesis were: to provide new insights into the complexity of the relationship between high environmental ammonia levels and disease susceptibility in fish; to test the hypothesis that ammonia acts through immunosuppression of the innate immune system; and also, to understand if cortisol mediates that suppression, using the zebrafish, *Danio rerio*, as the animal model.

In Chapter 2 the hypothesis that ammonia acts through immunosuppression of the innate immune system was tested, trying to provide new insights into the complexity of the relationship between HEA levels and disease susceptibility. To this end, the induction of a number of genes of the APR was analyzed by qPCR. Initially, LPS dose and time-course series were conducted to define experimental parameters to assess the effects of acute (24 h) and chronic (14 d) ammonia exposure on APR. Whole body cortisol levels in response to acute and chronic ammonia exposures were measured to establish if the treatment regime was eliciting organismal stress response.

In Chapter 3 the effects of acute and chronic HEA on response to *Edwarsiella tarda* infection were studied in zebrafish. To this end, they were challenged with intraperitoneal injection or immersion in a bacterial bath in the absence/presence of ammonia. Then, a number of acute phase response (APR) genes were analyzed on viscera (liver, intestine, pancreas and spleen) or gill by qPCR. In addition, the *E. tarda* LC50 (lethal concentration, 50%) value was assessed for fish exposed to ammonia versus control fish to see if zebrafish mortality was higher in the presence of HEA.

In Chapter 4 it was determined if cortisol mediated the environmental ammonia associated suppression of the acute phase response to LPS. To this end, two experimental studies were performed. In the first experiment, the effect of exogenous cortisol administration on the LPS-induced innate immunity related-genes was assessed in zebrafish. In the second experiment, the effects of glucocorticoid receptor blockade with mifepristone were evaluated in zebrafish exposed to LPS either in the presence or absence of high environmental ammonia.

Chapter 5 of this PhD thesis is the general discussion/integration of

results, with a final section on future perspectives.

CHAPTER 2

THE INHIBITORY EFFECT OF ENVIRONMENTAL AMMONIA ON *DANIO RERIO* LPS INDUCED ACUTE PHASE RESPONSE

Gonçalves, A. F., Páscoa, I., Neves, J. V., Coimbra, J., Vijayan, M. M., Rodrigues, P., Wilson, J. M., 2012. The inhibitory effect of environmental ammonia on *Danio rerio* LPS induced acute phase response. Dev. Comp. Immunol. 36 (2), 279–288.

Abstract

Ammonia is a toxic by-product of amino acid catabolism and a common environmental pollutant that has been associated with increased disease susceptibility in fish although the mechanism is not well understood. The hypothesis that elevated environmental ammonia acts by impairing the acute phase response was addressed (APR). Specifically, it was determined the impact of sub-lethal acute (24h) and chronic (14d) ammonia exposure on acute phase protein gene expression in zebrafish using a challenge with bacterial LPS (i.p 10µg/g after 24h). A panel of LPS responsive genes (*saa*, *hamp*, *lect2*, *hp* and *il1β*) was identified and evaluated by qPCR. Ammonia was found to impair induction of *saa*, *hamp* and *lect2* by 50-90%, providing evidence that ammonia may act potentially through immunosuppression. It was also determined that whole-body cortisol levels of fish subjected to acute (15min, 1h and 24h) and chronic (14d) HEA were higher compared with control fish. These results suggest that the elevated cortisol levels after HEA exposure may have been instrumental in this immunosuppression. Thus providing a mechanistic explanation for the association of increased disease susceptibility that is associated with environmental ammonia exposure.

Introduction

Ammonia is an unusual toxicant in that it is environmentally relevant and it is also produced naturally as a metabolic waste of amino acid catabolism within the organism. Ammonia is also released into the environment through the application of industrial fertilizers and input of biological wastes (Ip et al., 2004; USEPA, 1999). In aquaculture, ammonia toxicity may also be problematic during rearing stages in which fish are held at high densities with minimal water flow, or when using recirculating water systems, all of which may lead to elevated levels. In solution ammonia exists either as NH_3 or NH_4^+ in an equilibrium that is largely pH dependent ($NH_3 + H^+ \leftrightarrow NH_4^+$, pK 9.2). It is toxic to animals if accumulated in tissues and acute toxicity of ammonia is mainly due to its effect on the central nervous system in vertebrates (Randall and Tsui, 2002). Fish are generally ammonotelic and rely mainly on passive diffusion down the concentration gradient between the body and water for elimination. If the concentration in surrounding water exceeds that of blood (reverse diffusion gradient), it becomes difficult to remove, and conversion to nontoxic forms becomes a priority to avoid accumulation to toxic levels. Higher vertebrates make use of uric acid and urea (Wright et al., 1995). However, fish can tolerate higher internal levels of ammonia than mammals, but ammonia accumulation still leads to cell and animal death through its impairment of energy metabolism, by interfering in the Krebs cycle and, more importantly, through its interference with cellular ion and acid-base homeostasis (Ip et al., 2004). Exposure to high environmental ammonia (HEA) also causes gill hyperplasia, anemia, hypercortisolemia and ionoregulatory problems (USEPA, 1999; Ip et al., 2004; Wilkie, 2002). Ammonia causes convulsions, coma and death, probably because elevated NH_4^+ displaces K^+ and depolarizes neurons, causing activation of NMDA type glutamate receptor, which leads to an influx of excessive Ca_2^+ and subsequent cell death in the central nervous system (Randall and Tsui, 2002).

Ammonia is also generally accepted to increase susceptibility to different parasitic, bacterial and viral fish diseases (Carballo et al., 1995; Carballo and Munõz, 1991; Hanson and Grizzle, 1985). For example, juvenile Chinook salmon (*O. tshawytscha*) previously exposed to 0.12-0.49mM of total ammonia

nitrogen (TAN)[1] levels were more susceptible to *V. anguillarum* challenge (Ackerman et al., 2006). Also, infection caused by *Tetrahymena sp.* was significantly higher when guppies, *Poecilia reticulata* were exposed to 0.11-0.32mM TAN[1] (Pimenta Leibowitz et al., 2005). In addition, Walters and Plumb (1980) showed that Channel catfish, *Ictalurus punctatus*, injected with *Aeromonas hydrophila* and exposed to 0.11mM TAN[1] had significantly higher total bacterial counts than controls. However, the mechanism of action of ammonia remains largely unknown. Detrimental effects are not always observed with ammonia exposure. Lease and collaborators (2003) showed that none of the more traditional toxicity endpoints (growth, whole-body ion content and swimming performance) were significantly affected by range of TAN up to 1.71mM[1] in Lost River suckers. Also, TAN concentrations of 0.24mM[1] did not affect the antibody response to *Aeromonas salmonicida* in sunshine bass (Hrubec et al., 1996). Morris and collaborators (2006) even demonstrated that survival of *Deltistes luxatus* exposed to *Flavobacterium columnare* increased significantly as unionized ammonia concentrations increased from 0 to 0.72mM TAN[1].

The inflammatory response is a complex process that is an important component of the innate immune response. Induction of the acute phase response (APR) in fish is most frequently associated with the activation of the inflammatory response, typically in response to a pathogen challenge (Bayne et al., 2001). In mammals, induction of acute phase proteins (APPs) is mediated by Toll-like receptors (TLRs), pro-inflammatory cytokines and CCAAT/enhancer-binding proteins (C/EBPs). Upon infection, TLRs can recognize the microbial components thus sensing the invasion of pathogens (Akira and Takeda, 2004). Stimulation of these receptors finally results in the production of pro-inflammatory cytokines such as interleukin 1, interleukin 6, and TNFα, which are important inducers of APPs (Cohen, 2002). These cytokines in turn induce the production of some C/EBPs such as C/EBPB and C/EBPD, which are important transcription factors of many acute phase genes (Poli, 1998). The APPs are regarded as having general functions in opsonisation and trapping of micro-organisms and their products, activating the complement system, binding cellular remnants, neutralising enzymes, scavenging free haemoglobin and radicals and modulating the immune response (Gruys et al., 2005).

[1] Ammonia concentrations standardized to mM TAN at pH 8 using correction formulas provided in the ammonia water criteria manual (USEPA, 1999) to facilitate the comparison between different studies.

In fish, an innate immunity response is stimulated by injection with bacteria (Lin et al., 2007; Wiens and Vallejo, 2010). Also, immersion of fish in a bacterial suspension has been shown to in- duce the innate immune response (Harriff et al., 2007). However, the injection of bacteria is time-consuming and, in the case of immersion, the use of fish-pathogenic bacteria bears the risk of contaminations of fish culture. In contrast, the stimulation of the innate immune response by bacterial lipopolysaccharides (LPS) is an alternative approach. LPS are also termed endotoxins and are considered to be a major virulence factor, being responsible for lethal effects and clinical manifestations of diseases in humans and animals (Swain et al., 2008). LPS is a cell wall component found in most Gram-negative bacteria. However, some Gram-positive bacteria, such as *Listeria monocytogenes*, also possess endotoxin-like activity (Wexler and Oppenheim, 1979). Higher vertebrates are extremely sensitive to endotoxins, even at low doses, but lower vertebrates such as amphibians and fish are found to be resistant to endotoxic shock. Fish like *Oncorhynchus kisutch* and *Oncorhynchus mykiss* are reported to be resistant to *Escherichia coli* and *Aeromonas salmonicida* endotoxins in doses up to 80 mg/kg body weight (Wedemeyer et al., 1968).

The zebrafish, *Danio rerio*, is the species used in the present study and is a tropical, freshwater fish belonging to the Cyprinidae family. It is a popular aquarium fish and is one of the most widely used vertebrate models (Phelps and Neely, 2005; Van der Sar et al., 2004). Zebrafish have a fully developed immune system with both innate and adaptive immune responses, allowing studies that involve both systems (Trede et al., 2004) and is an emerging model species for the study of fish as well as human diseases (Kari et al., 2007).

In the present study the hypothesis that ammonia acts through immunosuppression of the innate immune system was tested, and also provides new insights into the complexity of the relationship between HEA levels and disease susceptibility. To this end, the induction of a number of genes of the APR by qPCR was examined. Initially, LPS dose and time-course series were conducted to define experimental parameters to assess the effects of acute (24 h) and chronic (14 d) ammonia exposure on APR. The genes *saa*, *lect2*, *hp*, *hamp*, *il1β*, *tnfα*, *il10*, *crp*, *tlr4a* and *litaf* were studied in zebrafish. Whole body cortisol levels in response to acute and chronic ammonia exposures were measured to establish if the treatment regime was eliciting organismal stress response in this species (Alsop and Vijayan, 2008; Fuzzen et al., 2010;

Ramsay et al., 2009).

Material and Methods

Animals

Adult *Danio rerio* (0.2–0.9 g body mass) were obtained from a local aquarium fish supplier and held at 26ºC in a 100 L aquarium with aerated Porto city tap water (Na+ 0.5 mM, alkalinity 50 mg/l CaCO3, pH 8). The tank contained an external filtration system supplemented with sterilization by ultraviolet irradiation and 10% of the water was changed daily. The fish were fed with commercial fish food (TetraMin, Tetra, Germany) four times per day on automatic feeders (Eheim 3538, Germany) and were reared on a 12 h light/dark cycle.

Experiment 1 – LPS dose response

In order to determine the optimal dose of LPS for the induction of innate immune system genes, zebrafish were injected with a range of concentrations of LPS. Forty-two zebrafish were anaesthetized (1:10,000 tricaine methanesulfonate, Alpharma, UK, pH 7.5, adjusted with NaHCO3) and intraperitoneally injected (n = 6) with LPS from E. coli (Serotype O55:B5, Sigma) at 50 pg/g, 1 ng/g, 50 ng/ g, 1 µg/g or 10 µg/g wet mass, or phosphate-buffered saline (PBS; 16.2mM Na_2HPO_4, 3.8mM NaH_2PO_4, 150mM NaCl, pH 7.3) vehicle alone (sham group). An injection volume of 10 µg/g was used. Another group with no injection served as the control. After 24 h, fish were sacrificed and sampled (see Section x) for gene expression analysis.

Experiment 2 – LPS time course response

In order to determine the time-dependent effects of LPS on the induction of genes of the innate immune system, two groups of twenty-eight zebrafish were anaesthetized, intraperitoneally injected with 10 µg/g LPS (infected group) or PBS (sham group) and fish from each group were sampled at 6, 12, 24 or 48 h after injection (n = 7). This dose was based on the results of Experiment 1.

Experiment 3 – acute ammonia and LPS

To elucidate the effects of ammonia exposure on the induction of innate immune response genes by LPS, fish were LPS or sham injected in the presence or absence of elevated water ammonia levels. Twenty-eight zebrafish were anaesthetized and received intraperitoneal injections of either PBS or 10 µg/g LPS. After that they were placed in 5 L plastic aquaria with ammonia concentrations of 0 (control) or 1mM NH4Cl pH 8.0 buffered with 10mM Tris, for 24 h. This concentration of ammonia is known to be sublethal (Páscoa et al., 2008). The ambient ammonia concentration was increased by the addition of a 1M of NH4Cl stock solution. After 24 h the fish were sampled (n = 7; see Section x).

Experiment 4 – chronic ammonia and LPS

Twenty-eight zebrafish were placed in 20 L glass aquaria with 0 (control) or 0.5mM NH_4Cl, pH 8.0 buffered with 10mM of Tris, for 14 days. After that time, fish were anaesthetized and received intraperitoneal injections of either PBS or 10 µg/g LPS and were sampled 24 h later (n = 7; see section bellow). Zebrafish were fed twice a day with commercial flake food until 24 h before the injection and were then fasted until the end of the experiment. The ambient ammonia concentration was increased by addition of a stock solution of 1M of NH_4Cl. The same stock solution was used throughout the study. Temperature, O2 concentration, pH and water ammonia concentration were measured daily and 25% of water was changed daily. The total ammonia nitrogen (TAN) in the control tank was 0.241 ± 0.039 mM and in the ammonia tank was 0.623 ± 0.036 mM. The fish appetite and behavior were normal throughout the experiment and the ammonia levels did not result in any mortality/morbidity.

Whole-body cortisol levels after ammonia exposure

The effects of both acute and chronic ammonia exposure on whole fish cortisol levels were measured in a separate set of fish. High environmental ammonia exposed or control fish were sampled at 15 min, 1 h, and 24 h acute and 14 d chronic exposure to HEA (1 and 0.5mM TAN, respectively). Conditions were identical as described above. In addition, it was included an acute handling disturbance group as a positive control group to measure cortisol stress response. Briefly, all fish were netted simultaneously, suspended in the air for 3 min, returned to the original tank for 3 min, and resuspended in the air

for an additional 3 min exactly as described previously (Ramsay et al., 2009). The stressed fish were returned to the original tank and allowed to recover for 15 min until they were sampled.

Sampling

The zebrafish from each aquarium were heavily anaesthetized (1:5000 tricaine methanesulfonate, pH 7.5, adjusted with $NaHCO_3$), weighed and standard length measured. Fish were then killed by decapitation on ice, with a transverse cut anterior to the pectoral fins. Viscera (that include liver, intestine, pancreas and spleen) were excised and kept in RNA later and remaining carcasses were snap frozen in liquid nitrogen and stored at -80 ºC for later analysis.

Zebrafish for whole body cortisol measurements were anaesthetized with buffered MS-222 (1:5000), blotted on paper towels to remove excess water, weighed and immediately frozen in liquid nitrogen. The samples were stored at -80 ºC.

Total RNA extraction and cDNA synthesis

Total RNA was isolated using a commercial silica based columns (Illustra RNAspin Mini RNA Isolation Kit, GE Healthcare, UK). All steps were performed according to the manufacturer's instructions, including the on-column treatment of isolated RNA with RNase-free DNase. RNA concentration and purity were assessed by UV spectrophotometry (Genova, Jenway, Dunmow, England) and integrity confirmed by electrophoresis (1.2% formaldehyde agarose gel; Mini-Sub Cell GT Cell, Bio-Rad, Hercules, CA, USA) and stored at -80 ºC until further use. The cDNA for all experiments was synthesized from 1 µg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol.

Primers

Primers for a suite of innate immunity associated genes were designed from gene sequences available in Ensembl (www.ensembl.org) and GenBank (http://www.ncbi.nlm.nih.gov/), or taken directly from published studies (Table 2.1). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and were initially tested for specificity by RT-PCR.

Table 2.1: Primer pairs (sense and anti-sense, respectively) for qPCR with predicted product size and original gene accession number. Primers with an asterisk (*) were designed with Primer3 and reference sources are given for the remainder.

Gene Name	GenBank Accession No.	Forward and reverse primer sequences (5'-3')	Product size (bp)	Ref
Ef1α	NM_131263	TGGGTGTTGGACAAACTGAA CAACACCACCAGCAACAATC	190	*
saa	NM_001005599.1	CGGGGTCCTGGGGGCTATTG GTTGGGGTCTCCGCCGTTTC	141	Lin et al., 2007
lect2	BC162786.1	TTCTACTTTTGGCTGTGCTA ACATCCTCTTTTTTGGTTAC	98	Lin et al., 2007
hp	XM_689364.3	TGATGCTACAGCCTCTACGG GTGTTCTGGAAGCCTGGATG	66	Lin et al., 2007
hamp	NM_205583.1	CACAGCCGTTCCCTTCATAC TCAGATGTTGGTTCTCCTGC	80	Lin et al., 2007
Il1β	AY340959	TGGACTTCGCAGCACAAAATG GTTCACTTCACGCTCTTGGATG	150	Van der Sar et al., 2003
Tnfα	AY427649	ACCAGGCCTTTTCTTCAGGT TGGTCATCTCTCCAGTCTAAGG	303	Van der Sar et al., 2003
Il10	AY887900.1	ACGAGATCCTGCATTTCTACTTG AGCTCCCCCATAGCTTTATAGAC	235	Watzke et al., 2007
Crp	NM_001114901.1	GGGTGGACGGTCAACGCAGT ACGGTGCCGCCAGGACGAAT	70	Lin et al., 2007
Tlr4a	NM_001131051	TTTGATCAACAATGGCTTGG GATTTGAGGAGTGCCGGATA	208	*
Litaf	NM_001002184.1	CAGCGCTGCAAAAATAATCA TTGGAACAGGCCCTTTATTG	293	*

RT-PCR and Quantitative real-time PCR

Semi-quantitative RT-PCR was performed with 1 μl of sample cDNA and DFS DNA polymerase from FINNZYMES (Espoo, Finland). The PCR profile was as follows: an initial denaturation of 2min at 94 ºC, followed by 30 s at 94 ºC, 30 s at 58 ºC, 15 s at 72 ºC for 40 cycles and 72 ºC for 5 min. Reactions were carried out in a MJ MINI Personal Thermal Cycler (Bio-Rad). PCR products were loaded onto 2% agarose gels in TBE (Tris-borate-EDTA) buffer and run at 80 V (Mini-Sub

Cell GT Cell, Bio-Rad). A 100 base pair DNA ladder was run on every gel to confirm expected size of the amplification products. Gels were stained with GelRed and images acquired with a Fujifilm LAS-4000 Mini luminescent image analyzer. Relative levels of mRNAs for innate immunity genes were quantified by real-time RT-PCR analysis using SYBR green on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Each cDNA sample was diluted and then 5 µl added to a reaction mix containing 10 µl of 2x iQ SYBR Green Supermix (Bio-Rad), and 250 nM of each primer in a total reaction volume of 20 µl. Each sample was prepared in duplicate for each gene. The cycling profile was the following: 94 ºC for 3.5 min, 40 cycles of 94 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 30 s. A melting curve was generated for every PCR product to confirm the specificity of the assays and a dilution series was prepared to check the efficiency of the reactions. *ef1*α was used as the housekeeping gene. The comparative CT method ($2^{-\Delta\Delta_{CT}}$ method) based on cycle threshold (CT) values was used to analyze the expression levels of the genes of interest.

Ammonia measurements

Whole body ammonia and water ammonia levels were measured using an enzymatic microplate technique, modified from Bergmeyer and Beutler (1985). Ammonia was initially extracted from fish carcasses. Briefly, 8% perchloric acid (PCA), 1:5 (w:v) was added to the tissue and after 10 min the samples were homogenized using a bead mill (6800 RPM 30 s Precellys24; Bertin, Montigny-le-Bretonneux, France). Homogenates were centrifuged (10,000g) for 15 min, at 4 ºC (Sigma refrigerated centrifuge 3K30 Sartorius Goettingen, Germany) and the supernatants were decanted and neutralized with saturated TRIS, and mixed. Carcass ex- tracts and untreated water samples were added in triplicate to a 96-well microplate on ice. Ammonia standards (0–350 µM) were also added in triplicate on each microplate. Reagent Mixture (11mM 2-Oxoglutarate, 0.56mM ADP, 155mM TEA, final concentrations in assay mixture) was added to each well, and thoroughly mixed for 2 min. NADH absorbance was read at 340 nm before and after adding 1.48 U GDH (Bio-Tek PowerWave 340 microplate reader, Vermont, USA). A linear regression was performed with the standards (ammonia concentration vs. difference in absorbance at 340 nm), and ammonia concentration of each sample was calculated through a linear equation.

Whole-body cortisol extraction and measurement

Whole zebrafish were homogenized using a bead mill in 1ml dH_2O (6800 RPM 2x 30 s Precellys24). Homogenates were centrifuged for 2 min at 14000 RCF at room temperature (Eppendorf MiniSpin Plus, Germany), and the supernatant was decanted and extracted with 5ml of diethylether, dried and resuspended in 100 µl of extraction buffer. Cortisol in the whole-body extracts of individual fish was measured using a Cortisol Elisa Kit (Neogen, USA) and all steps were performed according to the manufacturer's instructions following validation for use with zebrafish. Cortisol values were corrected for dilution factors and expressed per g of fish mass.

Statistics

The data are presented as means ± standard error of the mean (SEM). Sigma Stat (3.0 SPSS, Chicago, IL, USA) was used for all statistical analyses and $P < 0.05$ was considered significant. One-way analysis of variance followed by Student-Newman-Keuls post- tests were used to compare differences between means for Experiment 1. Two-way analysis of variance followed by Student-Newman-Keuls post-tests were used to compare differences for Experiments 2-4. T-tests were used to compare differences between whole-body cortisol levels for the handling stress and chronic ammonia exposure experiments, while a 2-way ANOVA followed by Student-Newman-Keuls post-test was used for the acute ammonia exposure experiment.

Results

Experiment 1 – LPS dose response

Expression of a panel of genes was evaluated for APR induction, in the viscera (liver, intestine, pancreas and spleen), 24 h after injection with a range of LPS doses (50 pg–10 µg/g), by real-time RT-PCR. Serum amyloid A (*saa*), leukocyte cell-derived chemotaxin 2 (*lect2*) and hepcidin (*hamp*) expression levels increased significantly with the highest concentration of LPS (10 µg/g; Fig. 2.1a, b and d), with no significant changes observed for lower doses, when compared to the control and sham groups. The expression of haptoglobin (*hp*) was also increased with 1 µg/g of LPS, but not with 10 µg/g LPS, due to the high variability of this group (Fig. 2.1c).

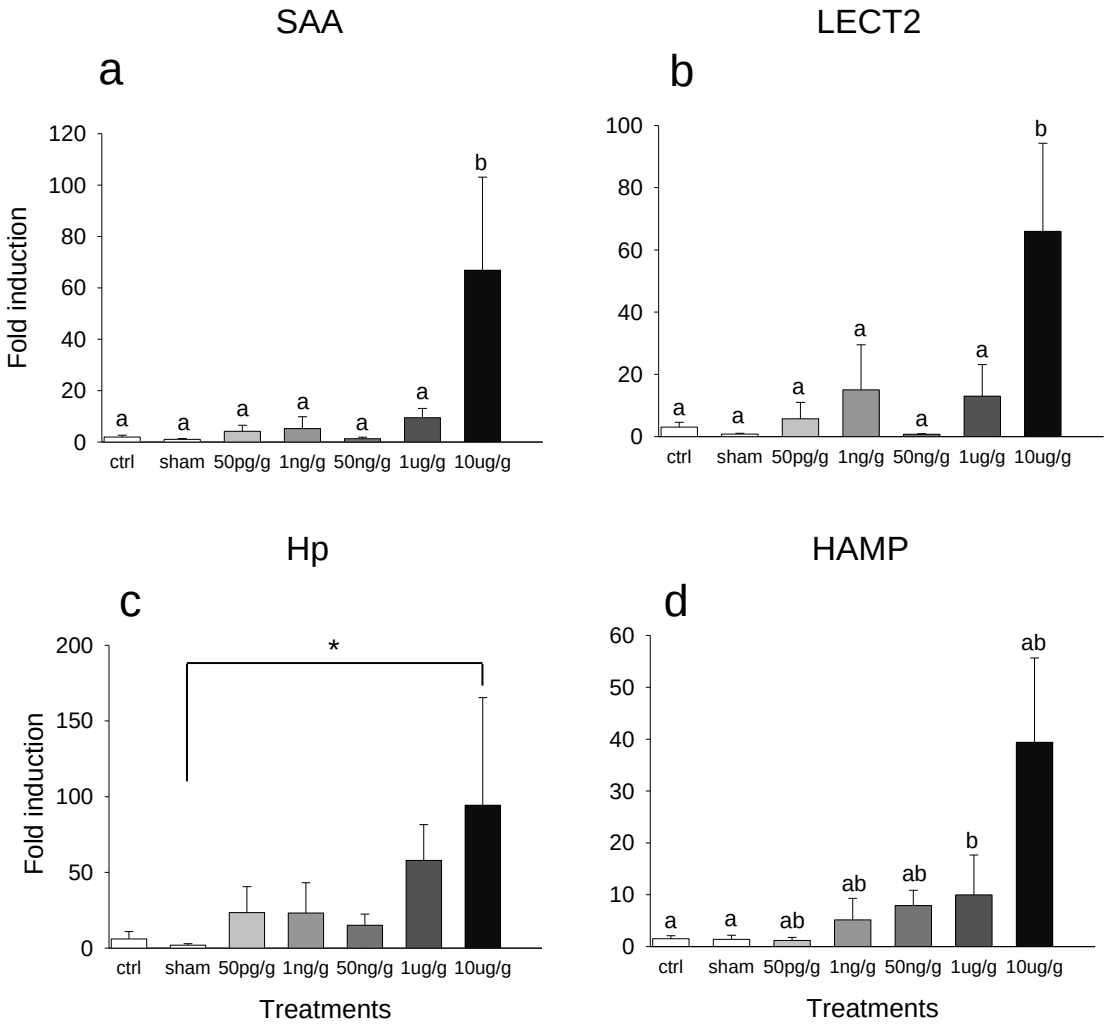

Figure 2.1. Fold induction in response to LPS dose of **a)** SAA; **b)** *lect2*; **c)** *hp*; and, **d)** *hamp* relative to *Ef1α*, determined by qPCR, in zebrafish viscera (liver, intestine, pancreas and spleen). Fish were i.p. injected with different doses of LPS, PBS (sham injected) or not injected (ctrl). Total RNA was extracted from fish that were sampled 24h post injection. Error bars represent SEM (n=6). Data analysed by 1-way ANOVA and *post hoc* Student-Newman-Keuls test. Bars with like characters are not significantly different.

Tumor necrosis factor a (*tnfα*), interleukin 1b (*il1b*), interleukin 10 (*il10*), C-reactive protein (*crp*), Toll-like receptor 4a (*tlr4a*), and LPS-induced TNFα (*litaf*) did not change significantly after exposure to the different doses of LPS (data not shown).

Experiment 2 – LPS time course response

Expression levels of genes of interest selected from experiment 1 were measured in the viscera (liver, intestine, pancreas and spleen) by real-time RT-PCR 6, 12, 24 and 48 h after injection with 10 µg/g LPS. A steady increase of *saa* was observed following LPS injection (Fig. 2.2a), with a peak of expression at 24 h after the injection, followed by a recovery to near control levels at 48 h. *lect2* expression followed a pattern similar to *saa* (Fig. 2.2b), with a gradual increase up to 24 h and a recovery to near control levels at 48 h. The expression of *hp* had an earlier transient increase 6 h and 12 h after the injection of LPS, decreasing at 24 h and returning to control levels at 48 h (Fig. 2.2c). *hamp* expression was higher for LPS than PBS and the induction was stronger for 6 h and 12 h compared with 24 h and 48 h (Fig. 2.2d).

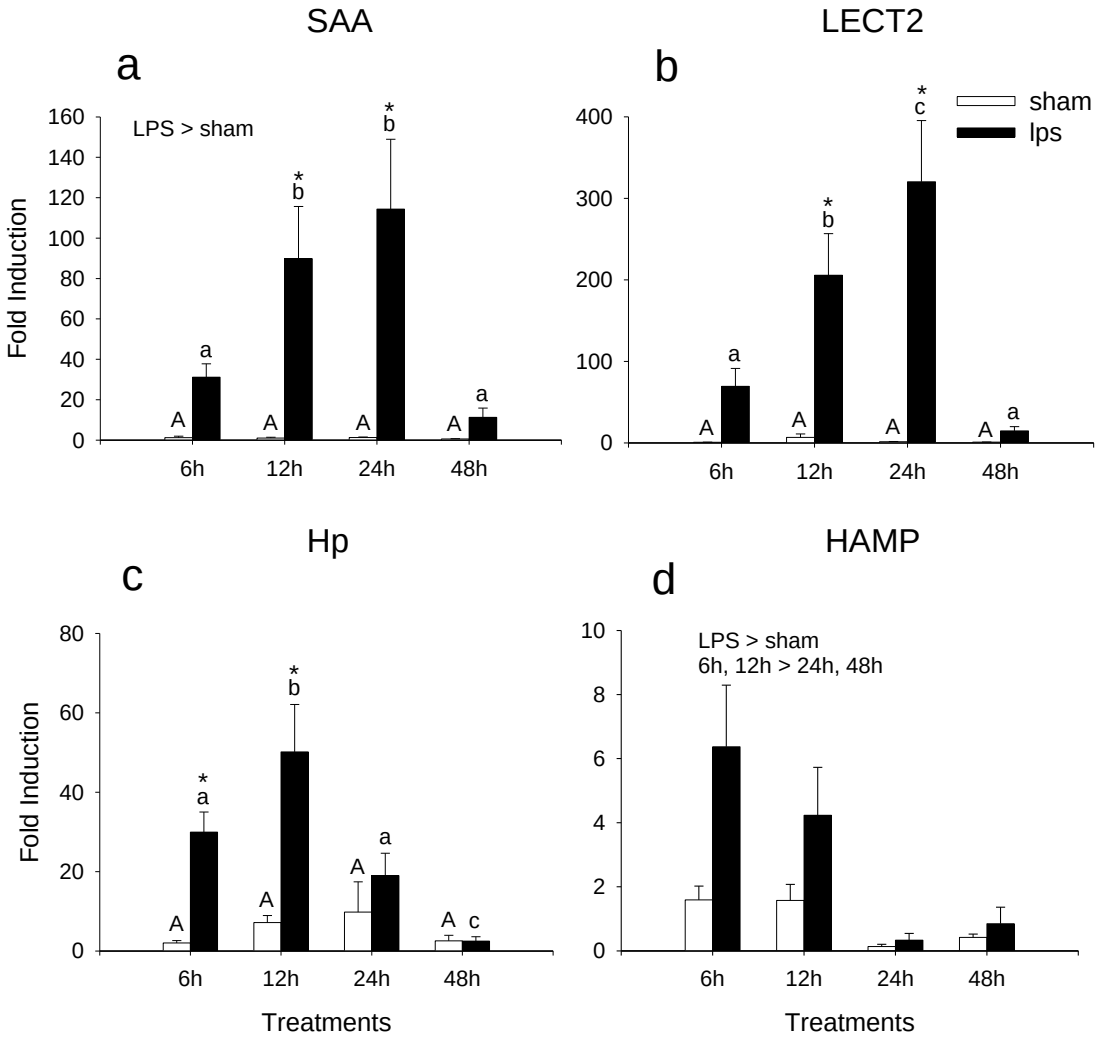

Figure 2.2. Time course fold induction in response to i.p. injection with 10µg/g LPS (black bars) compared to control fish (sham injected; white bars) as determined by qPCR for **a)** *saa*; **b)** *lect2*; **c)** *hp*; and, **d)** *hamp* relative to *ef1α*. Total RNA was extracted from

fish that were sampled at 6, 12, 24, 48h post injection. Error bars represent SEM (n=7). Data analysed by 2-way ANOVA and *post hoc* Student-Newman-Keuls test. Bars with like characters are not significantly different (sham upper case and LPS lower case characters). The asterisk (*) indicates a significant difference between the sham and LPS groups at a given time point.

The expression of *Il1β* was significantly elevated 6 h and 12 h after the injection with LPS. However, by 24 h it returned to control levels (Table 2.2). PBS-injected fish showed no significant changes in the expression of these genes.

Table 2.2: Data represent *il1β* time course response after injection of PBS or 10μg/g of LPS, by qPCR analysis. Mean ± standard error of the mean is show in the table (n=7). Values with like characters are not significantly different (PBS upper case and LPS lower case characters). The asterisk (*) indicates a significant difference between the sham and LPS groups at a given time point. Data analysed by 2-way ANOVA and *post hoc* Student-Newman-Keuls test.

Time	***il1β* Fold Induction**	
	PBS	**LPS**
6h	1.12 ± 0.21 (A)	13.40 ± 2.95 (a) *
12h	0.84 ± 0.14 (A)	7.45 ± 1.82 (b) *
24h	1.44 ± 0.41 (A)	2.96 ± 0.90 (b)
48h	1.67 ± 0.59 (A)	3.86 ± 1.32 (b)

Experiment 3 – acute ammonia and LPS

The effects of acute (24 h) sub-lethal ammonia exposure (1 mM) on LPS induction of APR was assessed in this experiment. The *saa* expression (Fig. 3a) was higher in LPS challenged fish and in fish that were not subjected to high environmental ammonia (HEA), levels were consistent with earlier experiments (Figs. 1 and 2). However, in HEA exposed fish the LPS induction of *saa* was significantly attenuated (~75%; Fig. 2.3a). HEA alone had no effect at *saa* levels in unchallenged fish. The *lect2* gene expression was significantly higher with LPS compared with PBS. No effect of acute HEA was observed (Fig. 3b). *hp* gene expression was significantly higher with LPS compared with PBS in animals that were not ex- posed to HEA, consistent with Experiments 1 and 2 (Figs. 2.1 and

2.2). *hp* gene expression increased in sham fish when they were subjected to HEA; however, further induction by LPS was completely blocked in the presence of HEA (Fig. 2.3c). *hamp* gene expression in- creased significantly in the LPS injected group that was not ex- posed to HEA, similar to Experiments 1 and 2 (Figs. 2.1 and 2.2). The increased gene expression induced by LPS was impaired when the fish were exposed to ammonia (Fig. 2.3d).

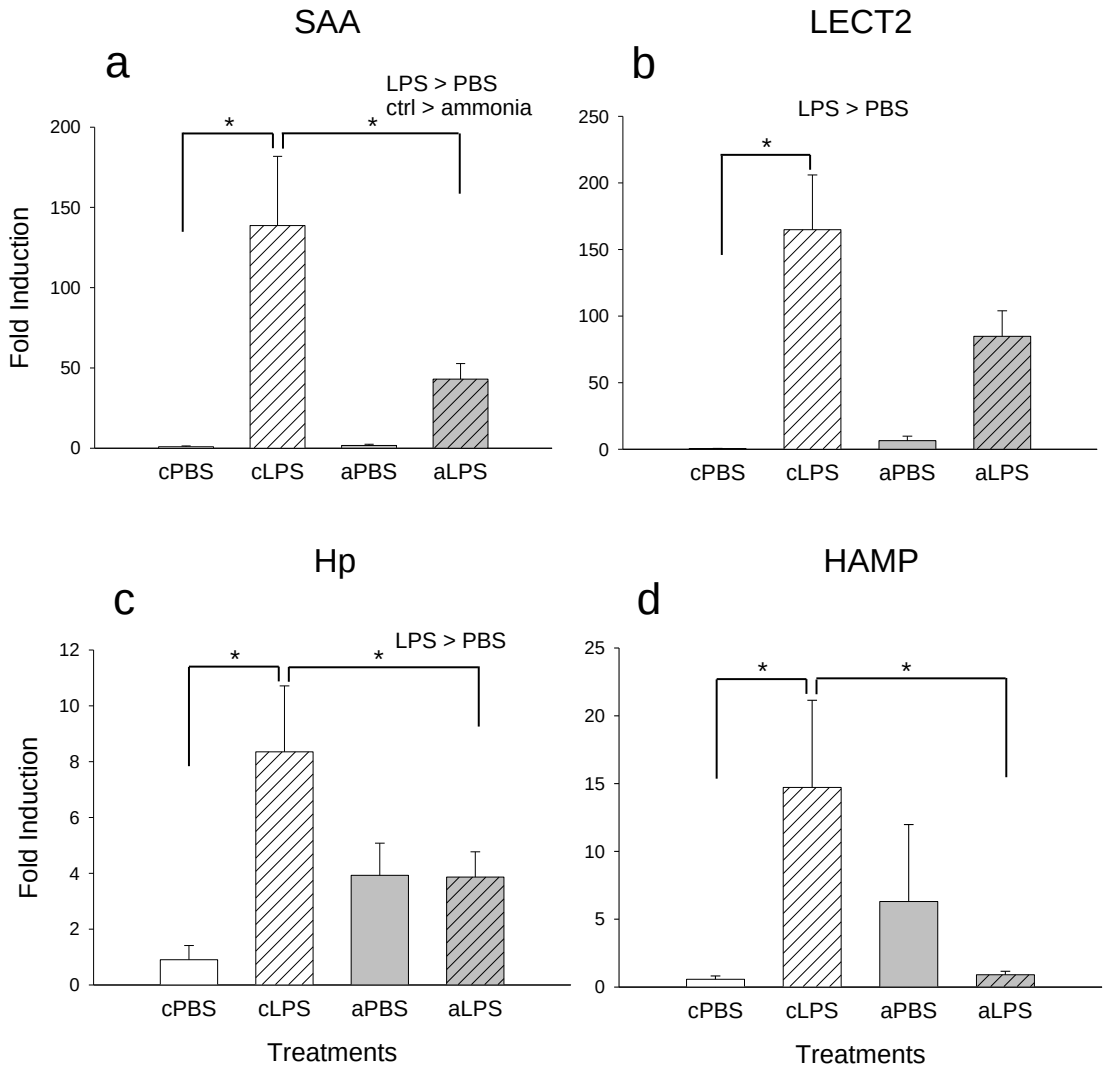

Figure 2.3. Effects of acute ammonia exposure on induction of innate immunity genes. Following an i.p. injection of 10µg/g LPS (hatched bars) or PBS fish were exposed to 1mM of NH_4Cl (pH 8) (grey bars) or control conditions and fold induction of **a)** SAA, **b)** *lect2*, **c)** *hp* and **d)** *hamp* relative to *Ef1α* measured after 24h. Error bars represent SEM (n=7). Data analysed by 2-way ANOVA and *post hoc* Student-Newman-Keuls test. Asterisks (*) indicate groups that are significantly different.

The whole body ammonia was 1.82 ± 0.24 μmol/g for the control fish injected with PBS with no statistical differences between treatment groups in this experiment. The whole-body cortisol levels of zebrafish subjected to acute ammonia were significantly higher at all sampling points. At 15 min, mean cortisol levels were almost 3-fold higher in ammonia exposed fish compared to controls. By 1 h, cortisol levels of the ammonia fish had declined but were still significantly greater from cortisol levels of control fish by almost 2-fold. Finally, at 24 h, mean ammonia cortisol levels were more than 3-fold higher com- pared to controls (Fig. 2.5b). As a positive control, cortisol levels of a net-stressed group were almost 14-fold higher than the control group (Fig. 2.5a).

Experiment 4 – chronic ammonia and LPS

The effects of chronic (14 d) sub-lethal ammonia (0.5 mM) exposure on LPS induction of ARP was assessed in this experiment. The *saa* gene expression was significantly higher with LPS com- pared with PBS, although no effect of chronic HEA was observed (Fig. 2.4a). LPS injection significantly increased *lect2* expression in both the absence and presence of HEA; however, in the latter group the increase was only 50% of control fish LPS induction (Fig. 2.4b). Chronic HEA in sham injected fish also resulted in greater expression of *lect2*. *hp* and *hamp* gene expression did not change significantly for any treatment (Fig. 2.4c and d).

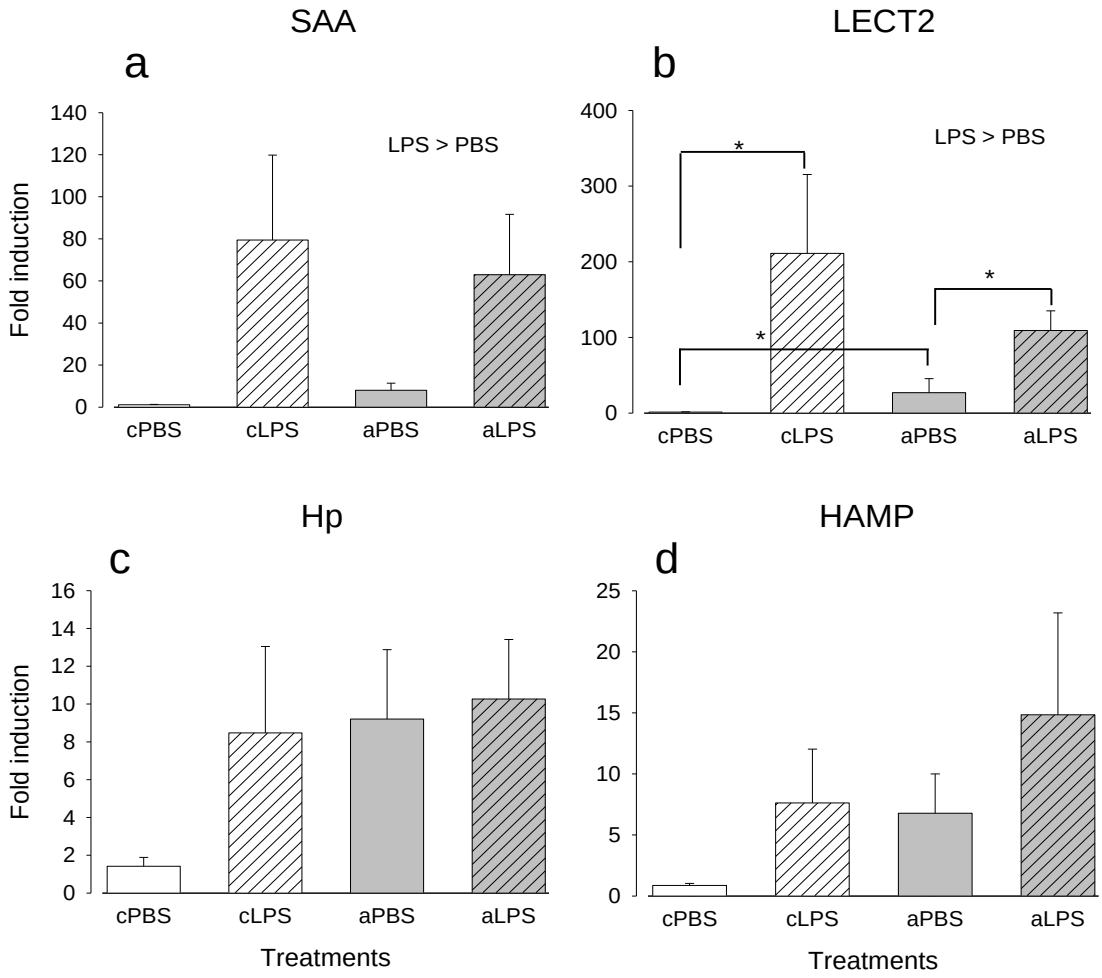

Figure 2.4. A comparison of the effects of 14 day pre-exposure to sub-lethal ammonia levels (0.5mM; grey bars) on the induction of innate immunity genes by 10µg/g LPS (hatched bars). Fold induction of **a)** *saa*; **b)** *lect2*; **c)** *hp*; and, **d)** *hamp* relative to *Ef1α* are shown. Error bars represent SEM (n=7). Data analysed by 2-way ANOVA and *post hoc* Student-Newman-Keuls test. Asterisks (*) indicate groups that are significantly different.

The whole body ammonia levels were 1.56 ± 0.06 µmol/g for the control fish injected with PBS, 1.86 ± 0.13 µmol/g for the control fish injected with LPS; 2.12 ± 0.05 µmol/g for the ammonia fish injected with PBS; and, 2.22 ± 0.01 µmol/g for the ammonia fish injected with LPS. The whole body ammonia levels were significantly higher in fish injected with LPS and fish exposed to HEA levels. Whole-body cortisol levels of zebrafish subjected to ammonia for 14 days were 2-fold higher compared with the parallel control group (Fig. 2 5c).

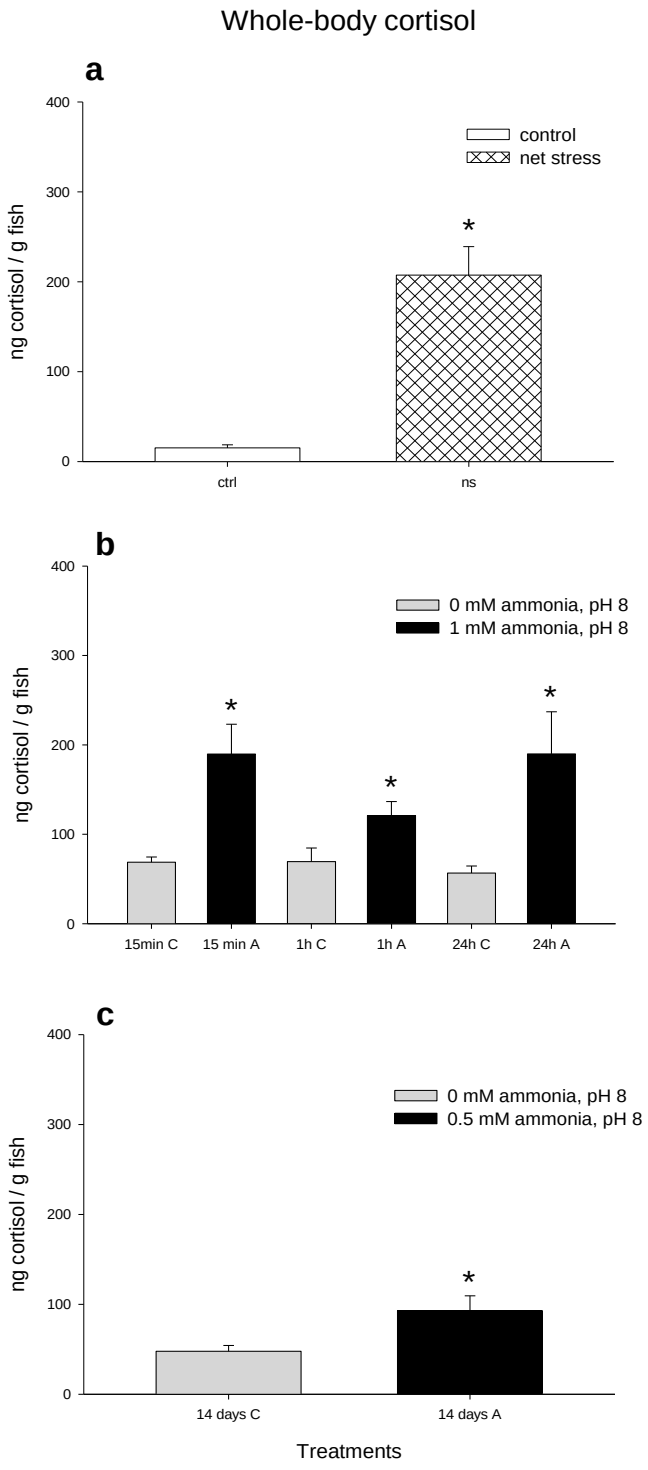

Figure 2.5. Mean whole-body cortisol (ng/g fish) levels of zebrafish of **a)** Control or net handling stress groups. **b)** Control or acute 1mM ammonia exposure after 15min, 1h or 24h, at pH 8. **c)** Control or chronic 0.5mM ammonia exposure (14 days), at pH 8. Error bars represent SEM (n=6). Data analysed by *t*-tests (a, c) or two-way ANOVA (b). Asterisks (*) indicate significant differences between treatment groups.

Discussion

In this study it was investigated whether sub-lethal acute and chronic ammonia exposure compromise the induction of acute-phase protein (APP) gene expression in adult zebrafish challenged with *E. coli* LPS. I present, to the best of my knowledge, the first direct evidence demonstrating that ammonia inhibits the induction of gene expression of *saa*, *hamp* and *lect2* in zebrafish, thus providing a clear link between elevated environmental ammonia levels and potential disease susceptibility. In addition, I were able to demonstrate that both acute and chronically elevated ammonia levels increased whole body cortisol levels, providing a possible mode of action by which ammonia maybe acting as an immunosuppressant.

LPS induction experiments

In the initial experiments a number of APPs that respond to LPS for subsequent experiments with ammonia were identified. These included SAA, LECT2, HAMP and Hp.

SAA is classified as a major APP because of the dramatic elevation of plasma SAA level in response to a variety of inflammatory conditions (Steel and Whitehead, 1994). The *saa* induction that was observed in zebrafish is in line with previous studies of challenges with LPS done in Atlantic salmon (Jorgensen et al., 2000) and rainbow trout (Villarroel et al., 2008). LECT2 was initially isolated as a possible chemotatic factor for neutrophils (Yamagoe et al., 1996) and subsequently demonstrated to be involved in liver regeneration (Sato et al., 2004), carcinogenesis and NKT cell homeostasis (Saito et al., 2004). However, the actual function in APR remains obscure. As expected the *lect2* induction by LPS seen in this study correlates with the work of Lin and colleagues (2007) that showed *lect2* induction upon infection of zebrafish with *A. salmonicida* and *S. aureus*. Hp, which circulates in the plasma, binds free hemoglobin with high affinity and inhibits its oxidative activity. In addition to this, the binding of Hp to receptors on effector cells mediates crucial leukocyte adhesion functions such as chemotaxis, phagocytosis and adhesion to endothelium, aggregation and cell-mediated cytotoxicity. In this study, I was observed an *hp* induction with LPS. This observation agrees with work done by Lin and colleagues (2007)

that showed an *hp* induction in zebrafish after infection with *A. salmonicida* and *S. aureus*. *hp* mRNA induction has also been observed in rainbow trout after exposure to *V. anguillarum*, by microarray analysis (Gerwick et al., 2007). HAMP inhibits the intestinal absorption (Laftah et al., 2004) and macrophage release (Knutson et al., 2005) of iron, providing an iron-restricted internal environment thus inhibiting bacterial growth in the serum (Nicolas et al., 2002). In this study, *hamp* gene expression increased with LPS, and this observation is consistent with studies of bacterial infection in sea bass (Rodrigues et al., 2006), zebrafish (Lin et al., 2007), and catfish (Bao et al., 2005). These results demonstrated that APPs in zebrafish may play an essential role in anti-infection as in other species. To test the role of pro-inflammatory cytokines in zebrafish APR, the mRNA levels of IL1b after exposure to LPS in a time course series were measured. The observed IL1b induction suggests that similar to the mechanism described in mammals (Lin et al., 2007), pro-inflammatory cytokines may play an early, pivotal role in induction of APPs in fish. The IL1b elevated response observed also correlates with experiments conducted previously with LPS or bacterial exposures in zebrafish (Novoa et al., 2009; Pressley et al., 2005; Lin et al., 2007). However, since the transient induction of IL1b gene expression occurred <24 h after exposure I did not measure expression in the experiments with HEA levels.

LPS-HEA effects and cortisol levels

In the acute study, I found that the presence of HEA blocked the LPS induction of SAA, *hp* and *hamp* gene expression by approximately 70%, 50% and 90%, respectively. In the chronic study, HEA affected the LPS induction of *lect2* by almost 50%. Also, *lect2* expression was higher in sham fish in the presence than in the absence of HEA. It is thus quite clear that ammonia exposure leads to an innate immune-suppression in these zebrafish as hypothesized.

It was observed that whole-body cortisol levels of fish subjected to acute and chronic HEA were higher compared with control fish. This suggests a modulation of the innate immune system response by elevated cortisol levels during HEA exposure in zebrafish. In contrast to other studies using acute stressors (handling, vortex), where elevated whole body cortisol levels were rapidly brought back to pre-stress levels (Ramsay et al., 2006; Fuzzen et al., 2010), continuous HEA in this study resulted in a persistent elevation of

cortisol.

It is known that the primary response of fish to stress is the production of catecholamine and cortisol by the interrenal gland (Harris and Bird, 2000) and there are studies showing that fish exposed to ammonia had significantly higher plasma cortisol levels (Ackerman et al., 2006; Carballo et al., 1995). Also, Ramsay and colleagues (Ramsay et al., 2006, 2009) showed that whole-body cortisol levels of zebrafish is an indicator of crowding stress and acute net handling stress. Varsamos and colleagues (2006) showed that temperature stress during early life stages of seabass (*Dicentrarchus labrax*) increased plasma cortisol concentrations and susceptibility to nodavirus.

Suppressive effects of cortisol have been reported on both specific and non-specific components of the immune system (Harris and Bird, 2000). The mechanisms of cortisol-induced suppression of the fish immune system are not yet clearly understood, but increasing evidence suggests that cortisol may act to inhibit IL-like factors (Engelsma et al., 2002). In fish, increases in cortisol have been demonstrated to inhibit the production of pro-inflammatory cytokines (Castro et al., 2011; Engelsma et al., 2002; Zou et al., 2000). Because induction of APPs is mediated by pro-inflammatory cytokines, I hypothesize that increases in cortisol will lead to inhibition of APPs production. Comparison of two lines of *Salmo salar*, selected for high and low cortisol response, showed the former to have reduced non-specific immunity and higher susceptibility to pathogens (Fevolden et al., 1993)

Consequently, higher cortisol content observed in the HEA group in the present study suggests the activation of the corticosteroid stress axis as a possible mechanism for the observed immunosuppression. The lack of a consistent change in chronic HEA effect on APPs (LPS-induced change noted only for one out of four analyzed), despite significant elevations in whole-body cortisol levels after 14 d, suggests other factors, in addition to cortisol, including altered tissue responsiveness to this hormone may be involved, but this remains to be established.

It is well documented that both natural and anthropogenic environmental stressors impair the homeostatic equilibrium in fish and affect physiological and immune functions (Sunyer et al., 1995; Tort et al., 1996). Fish in aquaculture experience different stressors; that are reported to suppress both the innate and adaptive immunity and exposure to these stressors has been suggested to be a factor predisposing animals to infectious diseases (Bly et al.,

1997). Challenges with various (bacterial and protozoan) pathogens have been used to assess the effects of different stressors. Acute stress increased the mortality of *Oncorhynchus mykiss* inoculated with *A. salmonicida* (Angelidis et al., 1987). When *Oncorhynchus tshawytscha* were challenged with *V. anguillarum* mortality increased following a transport stressor (Maule et al., 1989), and fish reared at high density displayed higher mortality when challenged with *Flavobacterium psychrophilum* (Iguchi et al., 2003).

In this study I found higher *hp* and *lect2* expression in sham fish following acute and chronic sub-lethal HEA exposure, respectively. The multiple and varied immunoregulatory effects of *hp* may provide an explanation for the up-regulation of this acute phase gene during periods of both invasive and non-invasive stress (Talbot et al., 2009), in this case, the stressor being the presence of ammonia. However, the explanation for the observed effect on *lect2* remains obscure.

In the chronic experiment, the whole body ammonia levels were significantly higher in fish subjected to HEA levels and fish injected with LPS. The increase in whole body ammonia with high concentrations of environmental ammonia can be due to retention of endogenous ammonia and/or uptake of exogenous ammonia (Ip et al., 2004). The higher whole body ammonia in fish injected with LPS may be associated with an increased metabolic rate of fish as they attempt to fight infection (Jones et al., 2007).

Conclusions

This study demonstrated that both acutely and chronically elevated environmental ammonia levels are capable of suppressing the induction of SAA, *hamp* and *lect2*, which are part of the acute phase response. Given the role of corticosteroids in immune suppression, the elevated cortisol levels in response to HEA provide a mechanistic explanation for the increased disease susceptibility that is associated with environmental ammonia exposure. This is of particular relevance to monitoring of environmental health (wild fish populations faced with pollution) and maintenance of fish under culture conditions (aquaculture).

CHAPTER 3

THE EFFECTS OF HIGH ENVIRONMENTAL AMMONIA ON ZEBRAFISH RESPONSE TO *EDWARDSIELLA TARDA* INFECTION

Abstract

High environmental ammonia (HEA) levels have been associated with increased disease susceptibility in fish, although the mechanism is not well understood. The hypothesis tested was that acute (24h) and chronic (14 days) HEA levels in zebrafish increase susceptibility to *Edwarsiella tarda* infection via immersion and injection challenge. To this end, a panel of candidate genes was chosen for evaluation of acute phase response induction, in gill and viscera, by real-time qPCR, after challenges. These genes included: hepcidin (HAMP), leukocyte cell-derived chemotaxin 2 (LECT2), serum amyloid A (SAA), haptoglobin (Hp), defensin 1, intestinal alkaline phosphatase (Iap) and complement component 3b (C3b). HEA was found to decrease survival during *E. tarda* challenge and impair bacterial induction of *hamp*, *lect2*, *saa*, *hp* and *c3b* by 40-90%. These results reveal that exposure to HEA suppresses the innate immune response to bacterial infection in fish providing a clear link between ammonia exposure and disease susceptibility.

Introduction

Ammonia is an unusual toxicant in that it is produced by, as well as being toxic to animals. It is produced naturally as a metabolic byproduct of amino acid catabolism and, also, it is environmentally relevant. Ammonia is produced as a result of the decomposition of organisms and sewage by microorganisms, the release of fertilizers, industrial emissions, and volcanic activity (Randall and Tsui, 2002). The major concern regarding ammonia toxicity is in aquatic systems, in regions of high human habitation and/or large numbers of farm animals, because urban and agricultural runoff and most biological waste are released into rivers and oceans (Randall and Tsui, 2002). In aquaculture, ammonia toxicity is problematic when using recirculating water systems, or during rearing stages in which fishes are held at high densities with minimal water flow, which may lead to high levels of ammonia. Ammonia has been shown to have significant adverse neurological and physiological effects in vertebrates. It is toxic to fishes (Randall and Tsui, 2002; Ip et al., 2004) as in other animals, causing convulsions, coma and death, probably because elevated NH_4^+ displaces K^+ and depolarizes neurons, causing activation of NMDA type glutamate receptors, which leads to an influx of excessive Ca^{2+} and subsequent cell death in the central nervous system (Randall and Tsui, 2002). Exposure to high environmental ammonia (HEA) levels also causes ionoregulatory problems, hypercortisolemia, anemia and gill hyperplasia (USEPA 1999; Wilkie 2002). It is generally accepted that ammonia increases disease susceptibility to various viral and bacterial diseases in fishes (Ackerman et al., 2006; Pimenta-Leibowitz et al. 2005; Carballo et al., 1995; Carballo and Muñoz, 1991). However, the mechanism of action of ammonia remains largely unknown and detrimental effects are not always observed with HEA exposure (Morris et al., 2006). In a previous study, I investigated whether sub-lethal acute and chronic ammonia exposure compromised the induction of acute-phase protein (APP) gene expression in zebrafish injected with *E. coli* LPS (lipopolysaccharide) and I showed, for the first time, that ammonia has an inhibitory effect on *Danio rerio* LPS induced acute phase response. High environmental ammonia-mediated elevation of cortisol levels may be playing a key role in this immune suppression (Gonçalves et al., 2012).

The zebrafish, *Danio rerio*, is a popular aquarium fish and is one of the most widely used vertebrate models and recently has been recognized as a valuable model for studying infection, disease and immunity (Phelps and Neely, 2005). In this regard, several research groups have identified pathogens that can infect and cause disease in zebrafish (Pressley et al., 2005; Neely et al., 2002; Davis et al., 2002; Prouty et al., 2003).

In this work zebrafish were challenged using the fish pathogen *Edwarsiella tarda*, a member of the Enterobacteriacae family, that was chosen because it grows at temperatures suitable for zebrafish maintenance and it has been established as a significant pathogen in a variety of fish species. The species most commonly associated with infection with this bacterium are *Ictalurus punctatus* (channel catfish), *Anguilla japonica* (Japanese eel) and *Paralichthys olivaceus* (Japanese flounder; Pressley et al., 2005). *Edwarsiella tarda* causes Edwardsiellosis, a generalized septicemia in a broad range of hosts, including humans, birds, reptiles, amphibians and aquatic mammals, in addition to fishes (Ling et al., 2001). Natural infection of fish is through waterborne contact with *E. tarda*. Ling and colleagues demonstrated that the gastrointestinal tract, gills and body surface of fish were the sites of entry by histological and infection kinetics studies (Ling et al., 2001). In addition, *E. tarda* was chosen because Pressley and colleagues already showed that adult zebrafish were susceptible to challenge by intraperitoneal injection and immersion and determined that *E. tarda* infection induced a typical acute inflammatory response (Pressley et al., 2005).

Invasion by pathogens, tissue injury or trauma induce changes in the quantities of several macromolecules in animal body fluids. These changes comprise one aspect of the acute phase response (APR) which involves metabolic changes in several organ systems (Nicolas et al., 1987). The APR is induced by plasma-borne signals called pro-inflammatory cytokines such as IL-1, IL-6 and TNFα. One clear indication of the APR is the remarkable increase in the concentrations of many plasma proteins that are synthesized in hepatocytes, known as acute phase proteins (APPs; Uhlar and Whitehead, 1999). An acute-phase protein has been defined as one whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 25 percent during inflammatory disorders. The APPs function in a variety of defense-related activities, such as limiting the dispersion and killing pathogens, repair of tissue damage, inactivation of proteases and

restoration of the healthy (homeostatic) state that prevailed before the stimulus (Bayne and Gerwick, 2001; Gabay and Kushner, 1999; Gruys et al., 2005).

It is known that HEA levels cause gill damage (Smart, 1976; Lang et al., 1987) but it remains unclear if this damage causes an increased susceptibility to infection. Elevated ammonia concentrations have previously been shown to damage gills in other fish species (Smart, 1976; Lang et al., 1987).

The aim of this work was to study the effects of acute (24h) and chronic (14 days) HEA on zebrafish response to *Edwarsiella tarda* infection. To this end, they were challenged with intraperitoneal injection or immersion in a bacterial bath in the absence/presence of ammonia. Then, a number of acute phase response (APR) genes were analyzed on viscera (liver, intestine, pancreas and spleen) or gill by qPCR. The panel of genes evaluated were: hepcidin (HAMP), leukocyte cell-derived chemotaxin 2 (LECT2), serum amyloid A (SAA), haptoglobin (Hp), defensin 1, intestinal alkaline phosphatase (Iap) and complement component 3b (C3b).

Material and Methods

Bacteria and media

The *Edwarsiella tarda* strain (CECT 849) used for the experimental infections was kindly provided by Dr. Miguel Ramos, (Faculdade de Ciências, Universidade do Porto). The bacteria was routinely cultured to mid-logarithmic growth in tryptic soy broth (TSB) growth medium from glycerol stock cultures stored at -80ºC. Liquid cultures were grown overnight at 27ºC with shaking. Quantification of logarithmic culture growth was performed by spectrophotometry and by plating dilutions of the culture on tryptic soy agar (TSA). After measuring absorbance at 600 nm, bacteria were resuspended in TSB to a final concentration of 10^9 CFU/ml and used in the experimental infection challenges.

Animals

Adult zebrafish, *Danio rerio*, were obtained from a local aquarium fish supplier and held at 26ºC in a 100 L aquarium with aerated Porto city tap water (Na^+ 0.5 mM, alkalinity 50 mg/l $CaCO_3$, pH 8). The tank contained an external filtration system supplemented with sterilization by ultraviolet irradiation (UV nano V²ecton, TMC, UK) and 10% of the water was changed daily. The fish were fed with commercial fish food (TetraMin, Tetra, Germany) four times per day on automatic feeders (Eheim 3538, Germany) and were reared on a 12h light/dark cycle. All fish used in the infection experiments were transferred to an isolated aquarium, and acclimated for several days before bacterial challenge. Prior to experimental manipulation, they were anesthetized with 1:10000 tricaine methanesulfonate, pH 7.5, adjusted with $NaHCO_3$.

Determination of the *E. tarda* LC50 value in the presence or absence of HEA

LC stands for "Lethal Concentration". LC50 is the concentration which causes the death of 50% (one half) of a group of test animals.

Zebrafish were anesthetized and intraperitoneally injected with different doses of *E. tarda* (10^4, $5x10^4$, 10^5 or $5x10^5$ CFU) and control fish were injected with TSB. After recovery from anesthesia, fish were placed in tanks with or

without 1mM NH_4Cl pH 8.0 buffered with 10 mM Tris, for 72h. Tanks were observed at 6h intervals to assess mortality and behavior. Moribund fish were removed immediately. Fish were not fed during the experiment. Temperature, pH and water ammonia concentration was measured daily and 20% of the water in the aquaria was changed.

LC50 values were calculated with Trimmed Spearman-Karber program (TSK, version 1.5; Table 3.1).

Table 3.1: *Edwardsiella tarda* LC50 values in the presence or absence of 1 mM ammonia (pH 8.0) and their 95% confidence limit for different time points (h: hours). Dose of bacteria is in CFU.

Time-points	Without HEA	With HEA
24 h	Minimum required trim is too large SK is not calculable	1.05×10^7 (3.47×10^6 - 3.15×10^7)
48 h	5.00×10^7 (95% confidence limits are not reliable)	5.74×10^6 (4.06×10^6 - 8.13×10^6)
72 h	9.24×10^6 (3.65×10^6 - 23.37×10^6)	3.04×10^6 (1.23×10^6 - 7.51×10^6)

Acute ammonia and *E. tarda* infection

In two different experiments, zebrafish were infected with the bacteria *E. tarda* by immersion or intraperitoneal (i.p.) injection in the presence or absence of elevated water ammonia levels for 24h. In the immersion challenge, zebrafish were exposed to 10^7 CFU/mL *E. tarda* or to TSB (sham) in a total volume of 1 L for 4 h. In the injection challenge, the same number of fish was anaesthetized and received i.p. injection of either 10^4 CFU *E. tarda* suspension (10 µl) or 10 µl TSB (sham).

After the bacterial infection, fish were placed in 30 L glass aquaria with ammonia concentrations of 0 (control) or 1 mM NH_4Cl pH 8.0 buffered with 10 mM Tris, for 24 h. This concentration of ammonia is sub-lethal (Páscoa et al., 2008). The ambient ammonia concentration was increased by the addition of a 1 M of NH_4Cl stock solution. Fish were fasted during both experiments and temperature, pH and water ammonia concentration were measured in each

tank. After 24 h the fish were sampled (see Section 2.7).

Chronic ammonia exposure and *E. tarda* infection

In two different experiments, zebrafish were kept 14 days in the presence or absence of elevated water ammonia levels and infected with the bacteria *E. tarda* by immersion or i.p. injection. Zebrafish were placed in 30 L glass aquaria with ammonia concentrations of 0 (control) or 0.5 mM NH_4Cl pH 8.0 buffered with 10 mM Tris, for 2 weeks. The ambient ammonia concentration was increased by addition of a 1 M NH_4Cl stock solution. Every day, temperature, pH and water ammonia concentration were measured and 20% of the water in the aquaria was changed. Fish were fed twice a day with commercial flake food until 24 h before the bacterial challenge and were then fasted until the end of the experiment. The fish appetite and behavior were normal and the ammonia levels did not result in any mortality/morbidity. In the immersion challenge, the total ammonia nitrogen (TAN) was 0.199 ± 0.030 mM in the control tank and 0.622 ± 0.029 mM in the ammonia tank. In the injection challenge experiment, the TAN was 0.207 ± 0.037 mM in the control tank and 0.609 ± 0.035 mM in the ammonia tank.

In the immersion challenge, after 14 days in the presence or absence of HEA, zebrafish were exposed by static immersion to 10^7 CFU/mL *E. tarda* for a duration of 4 h, in a total volume of 1 L. Control fish were treated similarly without being exposed to the pathogen. After that, fish were moved to separate 30 L tanks and maintained for 24 h until they were sampled (see section below). In the injection challenge, after 14 days in the presence or absence of HEA, fish were anaesthetized and received i.p. injections of either 10^4 CFU *E. tarda* suspension or 10 µl TSB. After recovery from anesthesia, each group of fish was moved to 30 L tanks, and maintained for 24 h until they were sampled (see section 2.7).

Reisolation of bacteria

Selected zebrafish from the groups infected with *E. tarda* were euthanized in tricaine methanesulfonate, (1:5000, pH 7.5, adjusted with $NaHCO_3$) and homogenized in TSB using a bead mill (6800 2x30Precellys24; Bertin, Montigny-le-Bretonneux, France). The diluted homogenate was streaked on TSA plates and incubated overnight at room temperature. Control fish were similarly sampled.

Sampling

The zebrafish were heavily anaesthetized (1:5000 tricaine methanesulfonate, pH 7.5, adjusted with $NaHCO_3$), weighed and standard length measured. Fish were then euthanized by decapitation on ice, with a transverse cut anterior to the pectoral fins. Gill and viscera (that include liver, intestine, pancreas and spleen) were excised and kept in RNA later and remaining carcasses were snap frozen in liquid nitrogen and stored at -80ºC for later ammonia analysis.

Total RNA Extraction and cDNA synthesis

Total RNA was isolated using a commercial silica based columns (Aurum Total RNA Mini Kit, Bio-Rad, Hercules, CA, USA). All steps were performed according to the manufacturer's instructions, including the on-column treatment of isolated RNA with RNase-free DNase. Total RNA was quantified at 260/280 nm using a Nanodrop spectrophotometer (ND-1000; Thermo Scientific, Wilmington, DE, USA), integrity confirmed by electrophoresis (1.2% formaldehyde agarose gel; Mini-Sub Cell GT Cell, Bio-Rad) and stored at -80ºC until further use. The cDNA was synthesized from 1 µg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol.

Primers

Primers for a suite of innate immune-related genes were designed from gene sequences available in Ensembl (http://www.ensembl.org) and GenBank (http://www.ncbi.nlm.nih.gov/), or taken directly from published studies (Table 3.2). Primers were designed using Primer3 (Rozen and Skaletsky, 2000) and were initially tested for specificity by semi-quantitative RT-PCR.

Table 3.2: Primer pairs (sense and anti-sense, respectively) for qPCR with original GenBank accession number. Primers with an asterisk (*) were designed with Primer3 and reference sources are given for the remainder.

Gene Name	GenBank Accession No.	Forward and reverse primer sequences (5'-3')	Ref
ef1α	NM_131263	TGGGTGTTGGACAAACTGAA CAACACCACCAGCAACAATC	*
saa	NM_001005599.1	CGGGGTCCTGGGGGCTATTG GTTGGGGTCTCCGCCGTTTC	Lin et al., 2007

lect2	BC162786.1	TTCTACTTTTGGCTGTGCTA ACATCCTCTTTTTTGGTTAC	Lin et al., 2007
hp	XM_689364.3	TGATGCTACAGCCTCTACGG GTGTTCTGGAAGCCTGGATG	Lin et al., 2007
hamp	NM_205583.1	CACAGCCGTTCCCTTCATAC TCAGATGTTGGTTCTCCTGC	Lin et al., 2007
defensin 1	AM181358	AACATGAAGCCCCAGAGCAT GAAAACTGGAGCTCCTGATC	Zou et al., 2007
iap	NM_001014353.1	GCCCTCACACTGCCTCTCA GAAACCGTGGACACTCCCATT	Bates et al., 2007
c3b	NM_131243	CAGTGGGAATATGTTGGCATTG TTAGCTGCCCTTCATAACCTGTT	Rojo et al., 2007

Semi-quantitative and Real-time RT-PCR

Semi-quantitative RT-PCR was performed with 1 µl of sample cDNA and DFS DNA polymerase from FINNZYMES (Espoo, Finland). The PCR profile was as follows: an initial denaturation of 2 min at 94ºC, followed by 30 s at 94ºC, 30 s at 58ºC, 15 s at 72ºC for 40 cycles and 72ºC for 5 min. Reactions were carried out in a MJ MINI Personal Thermal Cycler (Bio-Rad). PCR products were loaded onto 2 % agarose gels in TBE (Tris-borate-EDTA) buffer and run at 80 V (Mini-Sub Cell GT Cell, Bio-Rad). A 100 base pair DNA ladder was run on every gel to confirm expected size of the amplification products. Gels were stained with GelRed and images acquired with a Fujifilm LAS-4000 Mini luminescent image analyzer.

Relative levels of mRNAs for immune-related genes were quantified by real-time RT-PCR analysis using SYBR green on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Each cDNA sample was diluted and then 5 µl added to a reaction mix containing 10 µl of 2× iQ SYBR Green Supermix (PerfeCTa SYBR Green SuperMix, Quanta Biosciences, USA), and 250 nM of each primer in a total reaction volume of 20 µl. Each sample was prepared in duplicate for each gene. The cycling profile was the following: 94ºC for 3.5 min, 40 cycles of 94ºC for 30 s, 60ºC for 30 s and 72ºC for 30 s. A melting curve was generated for every PCR product to confirm the specificity of the assays and a dilution series was prepared to check the efficiency of the reactions. *Ef1α* was used as the housekeeping gene. The comparative CT method ($2^{-\Delta\Delta CT}$ method) based on cycle threshold (CT) values was used to analyze the expression levels of the

genes of interest.

Ammonia measurements

Whole body ammonia and water ammonia levels were measured using an enzymatic microplate technique, modified from Bergmeyer and Beutler (Bergmeyer and Beutler, 1985). Ammonia was initially extracted from fish carcasses. Briefly, 8% perchloric acid (PCA), 1:5 (w:v) was added to the tissue and after 10 min the samples were homogenized using a bead mill (6800 RPM, 30s, Precellys24). Homogenates were centrifuged for 15 min at 10,000 g, 4ºC and the supernatants were decanted and neutralized with saturated TRIS, and mixed. Carcass extracts and untreated water samples were added in triplicate to a 96-well microplate on ice. Ammonia standards (0 to 350 µM) were also added in triplicate on each microplate. Reagent Mixture (2mM NADH, 11 mM 2-Oxoglutarate, 0.56 mM ADP, 155 mM TEA, final concentrations in assay mixture) was added to each well, and thoroughly mix for 2 min. NADH absorbance was read at 340 nm before and after adding 1.48 U GDH (Bio-Tek PowerWave 340 microplate reader, Vermont, USA). A linear regression was performed with the standards (ammonia concentration vs. difference in absorbance at 340 nm), and ammonia concentration of each sample was calculated through a linear equation.

Statistics

The data are presented as means ± standard error of the mean (SEM). Sigma Stat (3.0 SPSS, Chicago IL USA) was used for all statistical analyses and a $P<0.05$ was considered significant. Two-way analysis of variance followed by Student-Newman-Keuls post-tests was used to compare differences.

Results

Determination of the *E. tarda* LC50 value in the presence or absence of HEA

After i.p. injection with four different doses of bacteria, zebrafish mortality was monitored over 72 h, at 6h intervals. Zebrafish exposed to HEA levels were found to be more sensitive to *E. tarda* infection with a lethal concentration to 50% of the test organisms (LC50) value for 72 h three times lower for animals in control conditions (Table 3.1).

Under low environmental ammonia conditions, 72 h post infection, there was a survival rate of 70% at the lowest dose of *E. tarda* (10^4 CFU), 60% for 5 x 10^4 CFU *E. tarda,* 50% for 10^5 CFU *E. tarda,* and 0% for 5 x 10^5 CFU *E. tarda* (the highest dose; Fig. 3.1 a). The effects of infection were accelerated in the presence of elevated water ammonia levels (1 mM, pH 8). At 72 h post infection, the survival rate for the lowest dose of *E. tarda* (10^4 CFU) was 50%, for 5 x 10^4 CFU *E. tarda* was 30%, and there was no survival (0%) for the highest doses (10^5 and 5 x 10^5 CFU *E. tarda;* ; Fig. 3.1 b).

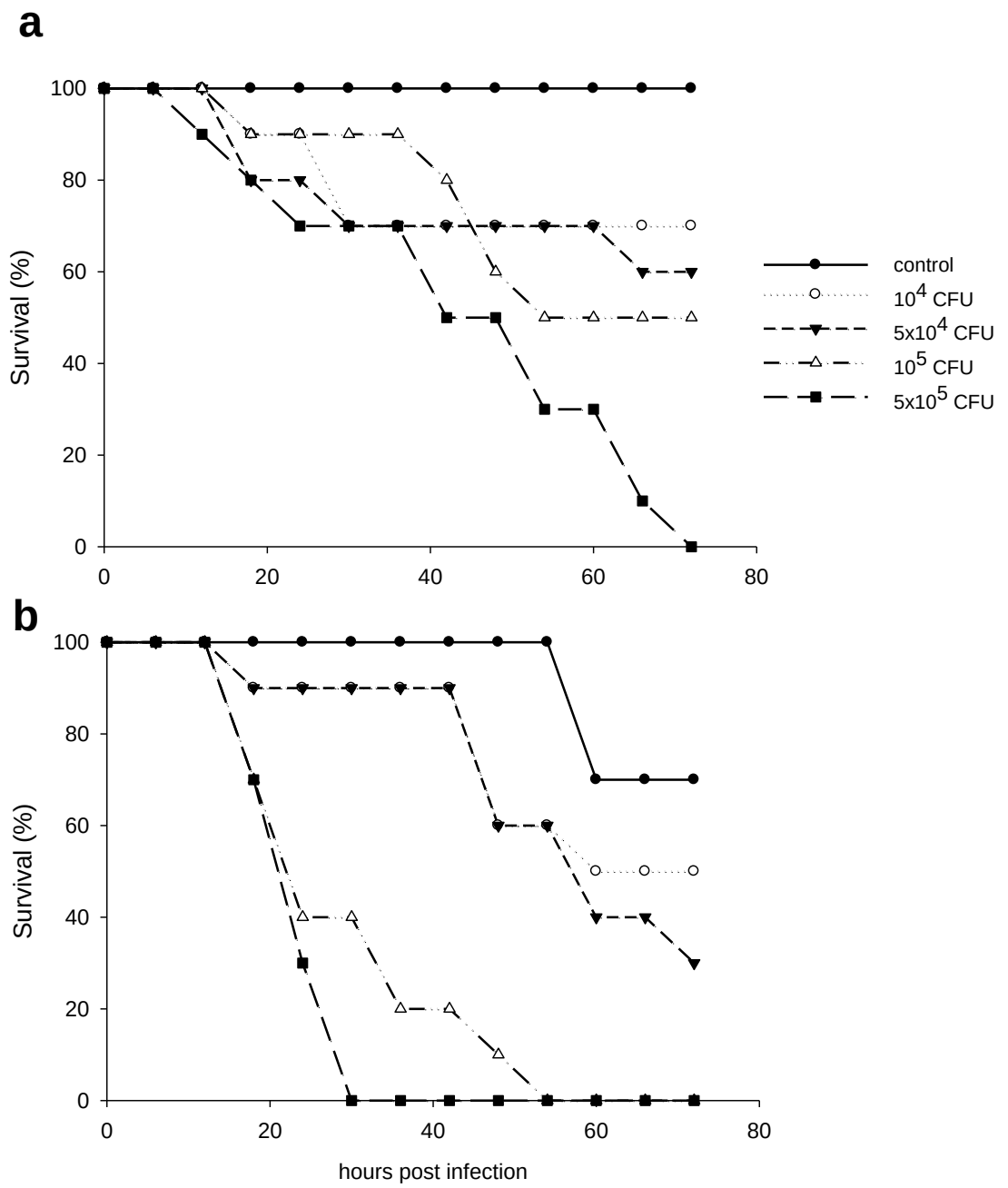

Figure 3.1. Percentage survival in zebrafish injected i.p. with different doses of *E. tarda* (10^4, 5 x 10^4, 10^5, 5 x 10^5 CFU *E. tarda* in 10ul TSB, control fish were injected with TSB alone). Fish were monitored during 72 h, in 6 h intervals **(a)** in the absence and **(b)** in the presence of elevated water ammonia levels (1 mM, pH 8).

Acute ammonia exposure and *Edwarsiella tarda* infection

The effects of acute (24 h) sub-lethal ammonia exposure (1 mM) on bacterial induction of innate immune-related genes were assessed in the two routes of infection (immersion and i.p. injection).

The *hamp* expression in viscera increased significantly in the *E. tarda*

immersion challenge in the control group that was not exposed to HEA, and that increased gene expression induced by the bacteria was impaired (~70%) when fish were exposed to HEA (Fig. 3.2 a). Bacteria injection also significantly increased visceral *hamp* expression in both the absence and presence of HEA; however in the latter group the increase was less than 40% (Fig. 3.2 b). In the gill, *hamp* gene expression was significantly higher in the fish challenged by *E. tarda* immersion compared with fish only exposed to TSB, although no effect of acute HEA was observed (Fig. 3.2 c). The *hamp* expression in gill was not significantly different with bacterial injection (Fig. 3.2 d).

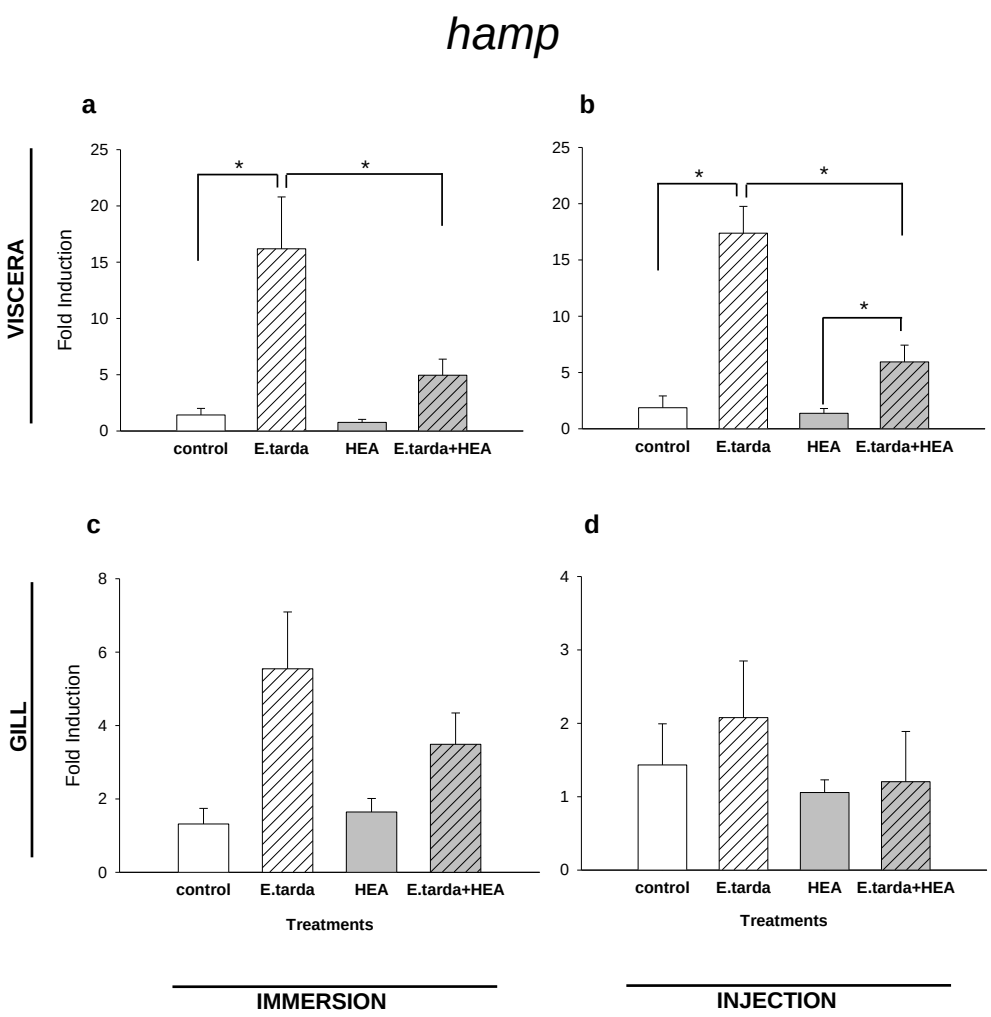

Figure 3.2. Effect of acute ammonia exposure on induction of *hamp* mRNA expression by *E. tarda* challenge via either i.p. injection or bath immersion. **(a, c)** Bacterial challenge by immersion with 10^7 CFU/mL *E. tarda* (hatched bars) or TSB. **(b, d)** Bacterial challenge by i.p. injection with 10^4 CFU *E. tarda* (hatched bars) or TSB. Following the bacterial challenge fish were exposed to 1 mM of NH_4Cl at pH 8.0 (grey bars) or control conditions (white bars) for 24 h. *hamp* was measured by q-PCR relative to *Ef1α* in **(a, b)** viscera and **(c, d)** gill. Data analyzed by 2-way ANOVA and post hoc Student-Newman-

Keuls test. Asterisks (*) indicate groups that are significantly different (n = 7).

In the immersion challenge, the *lect2* gene expression in viscera and gill was significantly higher in the fish challenged by *E. tarda* compared with fish exposed to TSB, although no effect of acute HEA was observed (Fig. 3.3 a, c). However, this may be due to the high variability in the low ammonia bacterial challenge groups. In the injection challenge, the *lect2* gene expression in viscera and gill was significantly higher with *E. tarda* compared with TSB in animals that were not exposed to HEA; however, the increased gene expression was impaired when the fish were exposed to ammonia (~80% and ~90%; respectively, Fig. 3.3 b, d).

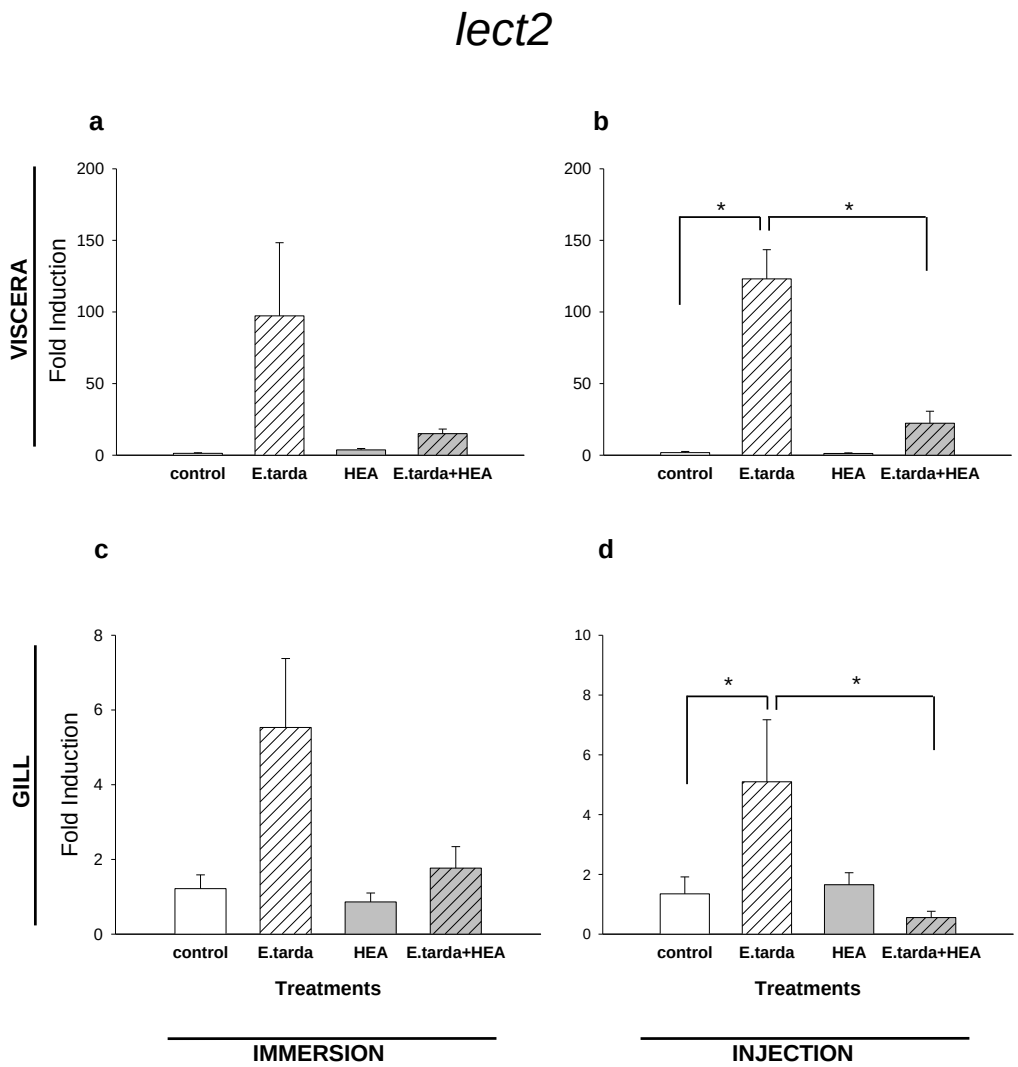

Figure 3.3. Effect of acute ammonia exposure on induction of *lect2* mRNA expression by *E. tarda* challenge via either i.p. injection or bath immersion. **(a, c)** Bacterial challenge by immersion with 10^7 CFU/mL *E. tarda* (hatched bars) or TSB. **(b, d)** Bacterial challenge by i.p. injection with 10^4 CFU *E. tarda* (hatched bars) or TSB. Following the bacterial challenge fish were exposed to 1 mM of NH_4Cl at pH 8.0 (grey bars) or control

conditions (white bars) for 24 h. *lect2* was measured by q-PCR relative to *Ef1α* in **(a, b)** viscera and **(c, d)** gill. Data analyzed by 2-way ANOVA and post hoc Student–Newman–Keuls test. Asterisks (*) indicate groups that are significantly different (n = 7).

In the immersion experiment, the *saa* expression in viscera was significantly higher for fish infected with bacteria compared with fish exposed to TSB, although no effect of acute ammonia exposure was observed (Fig. 3.4 a). In the same experiment, for the gill there were no significant changes for all treatments (Fig. 3.4 c). For the injection challenge, the *saa* expression in viscera was higher in bacteria challenged fish in low ammonia control conditions, however, in HEA exposed fish the bacterial induction of *saa* was significantly attenuated (~90%; Fig. 3.4 b). HEA alone had no effect on *saa* levels in unchallenged fish. For the gill, there was a higher induction of *saa* gene expression in fish injected with *E. tarda* than control fish, but the presence of HEA did not make any significant difference (Fig. 3.4 d).

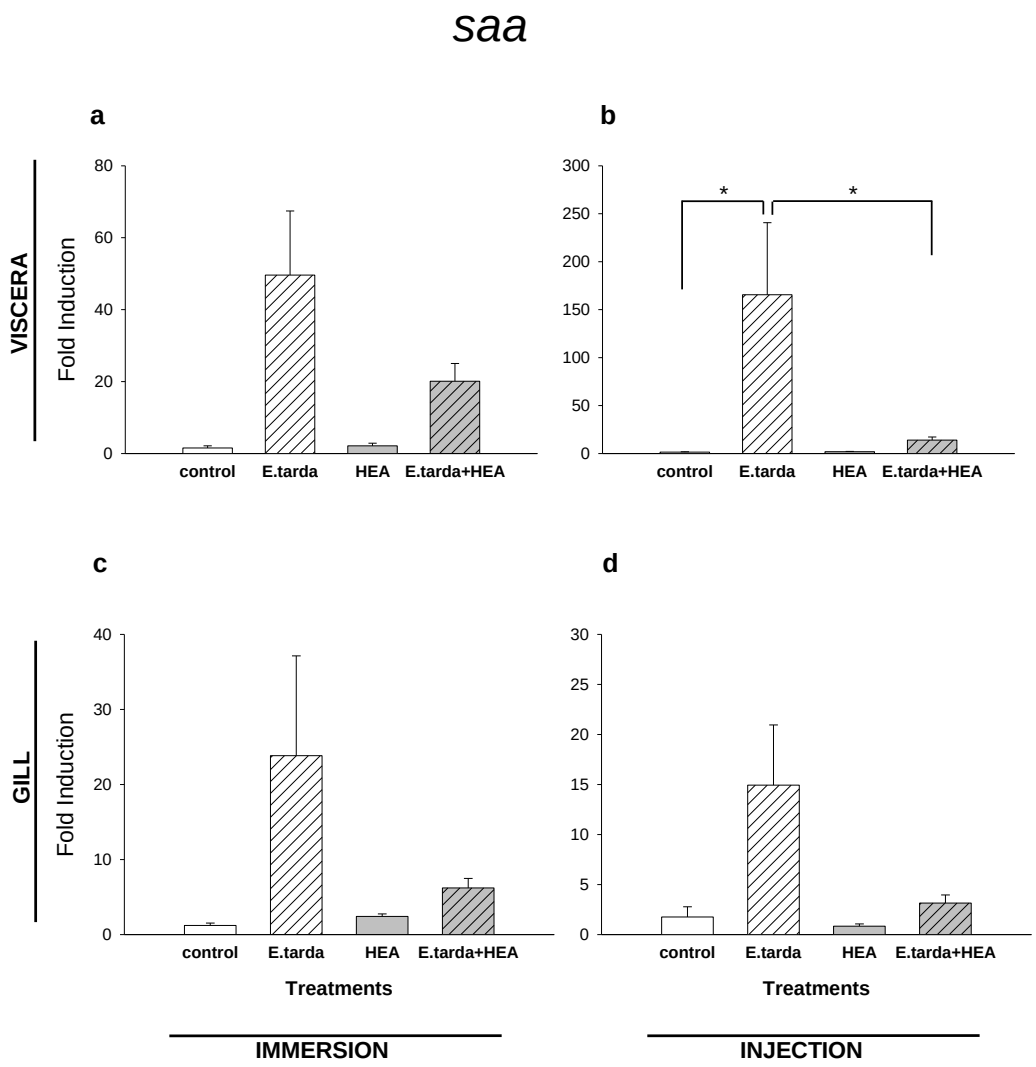

Figure 3.4. Effect of acute ammonia exposure on induction of *saa* mRNA expression by *E. tarda* challenge via either i.p. injection or bath immersion. **(a, c)** Bacterial challenge by immersion with 10^7 CFU/mL *E. tarda* (hatched bars) or TSB. **(b, d)** Bacterial challenge by i.p. injection with 10^4 CFU *E. tarda* (hatched bars) or TSB. Following the bacterial challenge fish were exposed to 1 mM of NH_4Cl at pH 8.0 (grey bars) or control conditions (white bars) for 24 h. *saa* was measured by q-PCR relative to *Ef1α* in **(a, b)** viscera and **(c, d)** gill. Data analyzed by 2-way ANOVA and post hoc Student–Newman–Keuls test. Asterisks (*) indicate groups that are significantly different (n = 7).

Bacterial immersion significantly increased *hp* expression, in viscera, in both the absence and presence of HEA; however in the latter group the increase was only 40% of control fish *E. tarda* induction (Fig. 3.5 a).The *hp* gene expression was higher for all groups of zebrafish infected with bacteria (by immersion or injection) compared with the groups subjected only to TSB and that gene induction was impaired by more than 60% in the presence of HEA, in

either gill or viscera (Fig. 3.5 b, c, d).

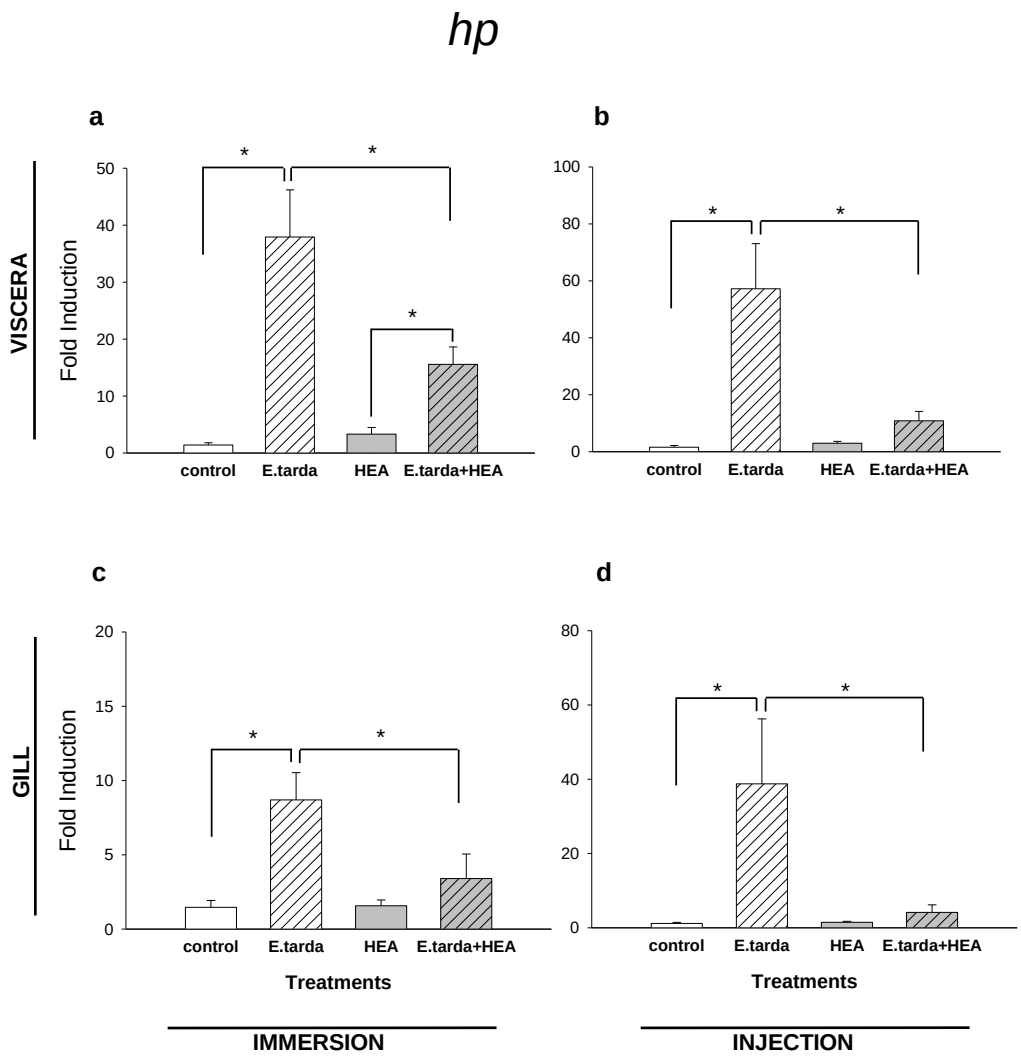

Figure 3.5. Effect of acute ammonia exposure on induction of *hp* mRNA expression by E. tarda challenge via either i.p. injection or bath immersion. **(a, c)** Bacterial challenge by immersion with 10^7 CFU/mL *E. tarda* (hatched bars) or TSB. **(b, d)** Bacterial challenge by i.p. injection with 10^4 CFU *E. tarda* (hatched bars) or TSB. Following the bacterial challenge fish were exposed to 1 mM of NH_4Cl at pH 8.0 (grey bars) or control conditions (white bars) for 24 h. *hp* was measured by q-PCR relative to *Ef1α* in **(a, b)** viscera and **(c, d)** gill. Data analyzed by 2-way ANOVA and post hoc Student–Newman–Keuls test. Asterisks (*) indicate groups that are significantly different (n = 7).

C3b gene expression in viscera increased significantly in the *E. tarda* immersion challenge in the groups that were not exposed to HEA, and that increased gene expression induced by the bacteria was 65% impaired when the fish were exposed to ammonia (Fig. 3.6 a). In the injection challenge, C3b expression in the viscera was higher in the fish challenged by *E. tarda*

compared with fish only exposed to TSB, although no effect of acute HEA was observed (Fig. 3.6 b). The C3b expression was not significantly different in gill, for all treatments (Fig. 3.6 c, d).

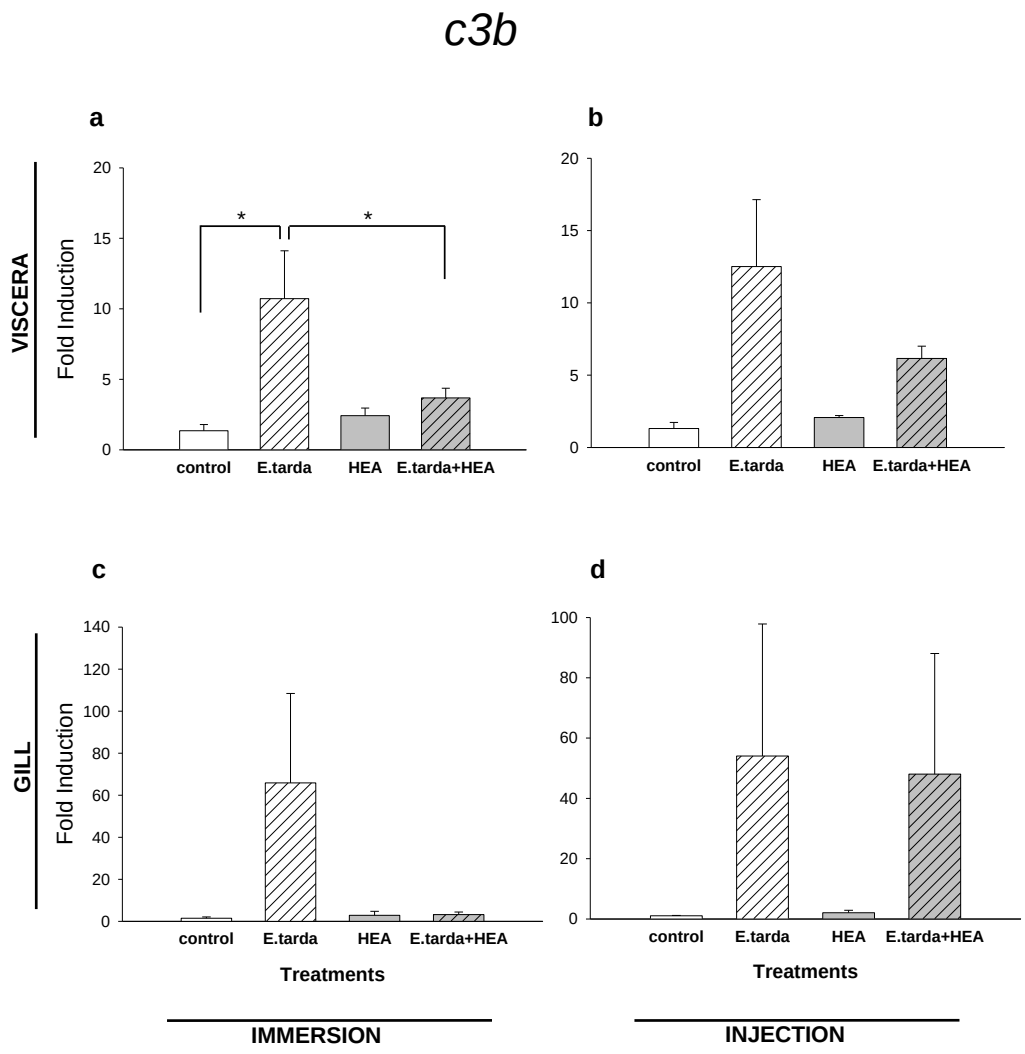

Figure 3.6. Effect of acute ammonia exposure on induction of *c3b* mRNA expression by *E. tarda* challenge via either i.p. injection or bath immersion. **(a, c)** Bacterial challenge by immersion with 10^7 CFU/mL *E. tarda* (hatched bars) or TSB. **(b, d)** Bacterial challenge by i.p. injection with 10^4 CFU *E. tarda* (hatched bars) or TSB. Following the bacterial challenge fish were exposed to 1 mM of NH_4Cl at pH 8.0 (grey bars) or control conditions (white bars) for 24 h. *c3b* was measured by q-PCR relative to *Ef1α* in **(a, b)** viscera and **(c, d)** gill. Data analyzed by 2-way ANOVA and post hoc Student–Newman–Keuls test. Asterisks (*) indicate groups that are significantly different (n = 7).

Whole body ammonia levels in acute HEA experiments

For the immersion challenge, the whole-body ammonia levels for the control group (fish immersed in TSB sham) was 1.62 ± 0.17 μmol/g with no statistical differences between all treatment groups. For the injection experiment, the whole-body ammonia levels for the control group (fish injected with TSB) was 1.79 ± 0.22 μmol/g, also with no statistical differences between treatment groups.

Effects of chronic ammonia exposure on *E. tarda* infection

The effects of chronic (14 d) sub-lethal ammonia (0.5 mM) exposure on bacterial induction of immune-related genes was studied by injection and immersion challenges with *E. tarda*. The doses of *E. tarda* were 10^7 CFU / mL in static immersion for a duration of 4 h or 10^4 CFU of bacteria injected i.p..

In the immersion challenge, the *saa* gene expression did not change significantly for any treatments or tissues (Fig. 3.7 a, c). Visceral *saa* expression was significantly higher with bacteria compared with no bacteria, although no effect of chronic ammonia was observed for the injection challenge (Fig. 3.7 b). For the same experiment, in gill, *saa* gene expression increased after injection with *E. tarda* in fish under low ammonia control conditions; however, that induction was 60% blocked in the presence of HEA (Fig. 3.7 d).

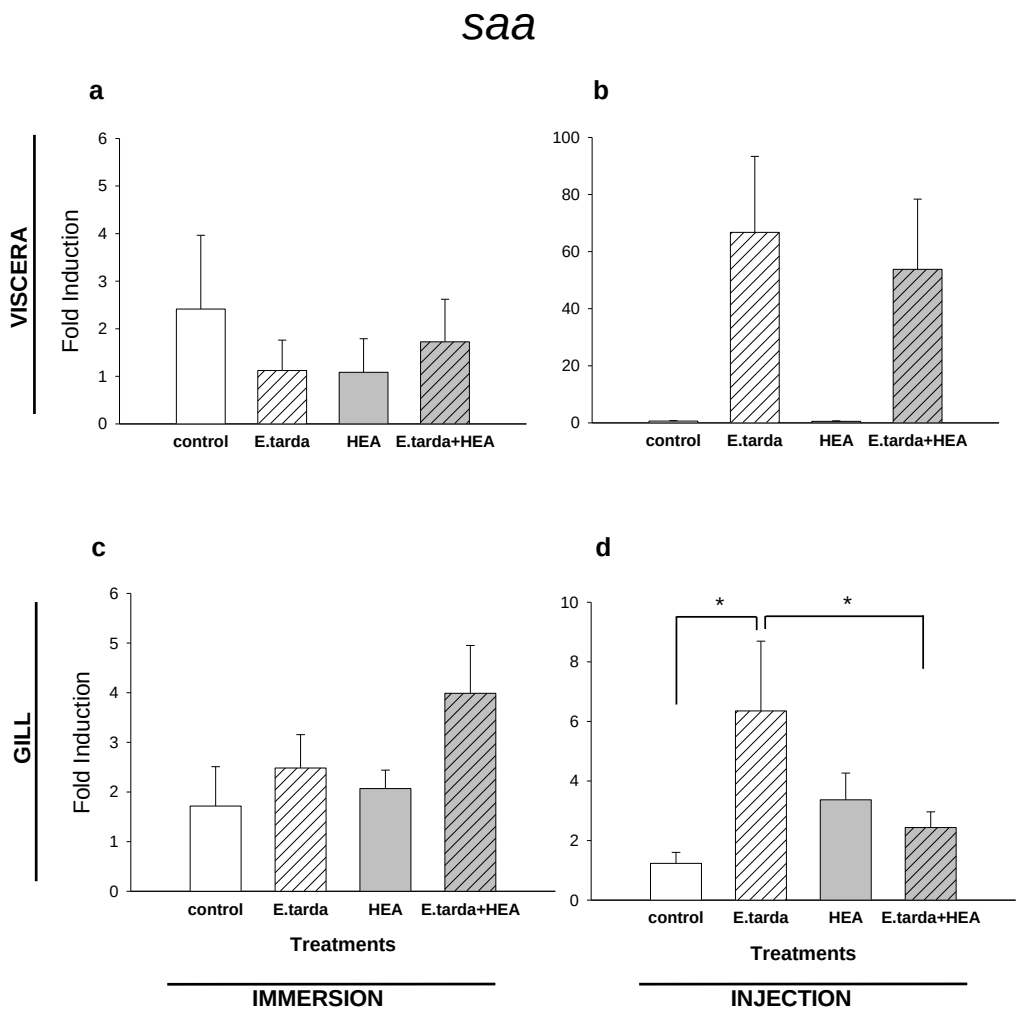

Figure 3.7. A comparison of the effects of 14 day pre-exposure to sub-lethal ammonia levels (0.5 mM at pH 8; grey bars) on the induction of *saa*. **(a, c)** By bacterial challenge by immersion in 10^7 CFU/mL *E. tarda* (hatched bars) or TSB. **(b, d)** Or bacterial challenge by i.p. injection with 10^4 CFU *E. tarda* (hatched bars) or TSB. Error bars represent SEM (n = 7). Data analyzed by 2-way ANOVA and post hoc Student–Newman–Keuls test. Asterisks (*) indicate groups that are significantly different.

lect2, *hamp*, *hp*, *Defensin 1*, *iap* and *c3b* gene expression did not change significantly for any treatment or tissue (data not shown).

Whole-body ammonia levels in chronic HEA experiments

In the case of infection by immersion, the whole-body ammonia for the control group (fish immersed in TSB) was 2.02 ± 0.18 µmol/g, not statistically different from infected fish. For the injection challenge, the whole-body ammonia for the control group (fish injected with TSB) was 2.23 ± 0.22 µmol/g,

not statistically different between the infected fish.

Discussion

In this study I investigated if acute (24h) or chronic (14 days) exposure to HEA levels affected the zebrafish acute phase response (APR) to *Edwarsiella tarda*. To this end, the induction of a number of genes of the innate immune response was examined in viscera or gill, after an infection challenge by immersion, to mimic natural infection conditions in fish, or by intraperitoneal injection which allows for a more controlled challenge.

It is well known that gill epithelium is critical to overall fish health as it is the primary site of O_2 uptake, ammonia excretion and ion regulation (Lease et al., 2003). Elevated ammonia concentrations have previously been shown to damage gills in other fish species (Lang et al., 1987; Carballo and Muñoz, 1991; Plumb, 1984), and this structural gill pathology has been linked to functional consequences in salmonids (Woodward et al., 1983). Several functional roles have been attributed to mucus in fish, including an important role in disease resistance (Shephard, 1994). Has been shown that HEA levels interfere with mucus renewal by retarding mucus production by the mucous cells (Lang et al., 1987) and reduce the presence of defensive substances in the mucus (Mock and Peters, 1990). These two factors together may facilitate bacterial colonization, which can be translated in higher fish mortality when in the presence of ammonia. In this study, it was observed that after i.p. injection with four different doses of *E. tarda* (10^6, 5 x 10^6, 10^7 and 5 x 10^7 CFU), zebrafish mortality due to bacterial infection was higher in the presence of elevated water ammonia levels (1 mM, pH 8) at 24h, 48h and 72 h post infection. I also realized that the *E. tarda* LC50 value for 72 h was 3 times lower for fish exposed to ammonia versus control fish. These results support the prediction that ammonia increases susceptibility to different bacterial and viral fish diseases (Carballo et al., 1995; Carballo and Muños, 1991; Hanson and Grizzle, 1985; Plumb, 1984).

In a previous study, I found that both short and long-term exposure to ammonia causes a significant increase in whole-body cortisol in zebrafish (Gonçalves et al., 2012); and, there are other studies showing that fish exposed to ammonia have significantly higher plasma cortisol levels (Ackerman et al., 2006; Carballo et al., 1995; Tomasso et al., 1981). These increases are

probably linked to an attempt to mobilize energy resources to cope with stress and maintain homeostasis.

It has been suggested that cortisol may act to inhibit IL-like factors (Engelsma et al., 2002) and have suppressive effects on both specific and non-specific components of the immune system (Harris and Bird, 2000). In fish, increases in cortisol have been demonstrated to attenuate the production of pro-inflammatory cytokines (Castro et al., 2011; Engelsma et al., 2002; Zou et al., 2000). In this study, gene expression analysis by real-time qPCR revealed that the presence of HEA had an immunosuppressive effect on the induction of a number of APPs that respond to infection, such as: hepcidin (*hamp*), leukocyte cell-derived chemotaxin-2 (*lect2*), serum amyloid A (*saa*), haptoglobin (*hp*) and complement component 3b (*c3b*), in the immersion and/or injection bacterial challenges. These APPs are well documented in various fish species (Boshra et al., 2006; Holland and Lambris, 2002; Lin et al., 2007; Rodrigues et al., 2006; Vilarroel et al., 2008). The inhibition of APP gene expression was present in viscera and in gill, although the fold induction was higher in the former. This likely happens because the changes in the concentrations of acute phase proteins are due largely to changes in their production by hepatocytes (Russel et al., 2006).

The function of most APPs has not been totally elucidated. However, the positive APPs are regarded as having general functions in opsonization and trapping of micro-organisms and their products, activating complement, binding cellular remnants like nuclear fractions, neutralizing enzymes, scavenging free haemoglobin and radicals, and modulating the host's immune response (Gruys et al., 2005).

Acute exposure to HEA produced a number of expression changes in several innate immune-related genes. Hepcidin is a small cysteine-rich protein with dual function as antimicrobial peptide and iron metabolism regulator (Rodrigues et al., 2006; Nicolas et al., 2002; Shike et al., 2002). In this study, there were significantly higher levels of hepcidin transcripts in gill of the infected fish in comparison to control animals; however, the HEA impact on induction was not significant. Conversely, in viscera, HEA impaired the bacterial induction of *hamp* by approximately 70% and 60%, both in the immersion and injection experiments, respectively. These results follow my previous study where the presence of ammonia blocked the LPS induction of *hamp* by almost 90% (Gonçalves et al., 2012).

Leukocyte cell-derived chemotaxin-2 (LECT2) is a multifunctional protein that acts as a chemotactic factor to neutrophils, involved in cell growth, differentiation and autoimmunity, with a role in liver regeneration (Sato et al., 2004), carcinogenesis (Overejo et al., 2004) and Natural killer T cell homeostasis (Saito et al., 2004). However, its actual function in the APR remains unclear. There are more studies that report a high induction of *lect2* upon infection, suggesting an important role of this gene in anti-infection (Lin et al., 2007). Real-time RT-PCR analysis performed on viscera and gill showed significantly higher expression in the fish injected with *E. tarda*, in comparison with the TSB alone group. It also showed that HEA levels had an inhibitory effect on that induction by 80% and 90%, respectively. In my previous study, the induction of *lect2* transcript level by LPS seemed to be diminished by ammonia; however no significant differences were found (Gonçalves et al., 2012).

Serum amyloid A (SAA) is thought to influence high-density lipoprotein-cholesterol transport. In tissues, it attracts inflammatory cells and inhibits the respiratory burst of leukocytes and modulates the immune response (Gruys et al., 2005; Gruys et al., 1994). The *saa* transcript levels in viscera increased after *E. tarda* injection, and that induction diminished 90% in the presence of ammonia. This correlates to my previous findings that ammonia blocks the LPS induction of *saa* by 70% (Gonçalves et al., 2012). *saa* transcripts were detected in zebrafish gill although in lower values compared with viscera; in agreement with the observation of Villarroel and colleagues, which showed expression of *saa* in skin, intestine and gills of rainbow trout infected with *F. psychrophilum* (Villarroel et al., 2008).

Haptoglobin (Hp) binds free hemoglobin released from erythrocytes with high affinity and thereby inhibits its oxidative activity (Wicher and Fries, 2006). In this study, *hp* was the gene that was most affected by the presence of ammonia. An impairment of *E. tarda* induction of *hp* was observed in both gill and viscera, in both immersion and injection challenges. In my previous findings HEA caused a 50% impairment of the LPS haptoglobin induction (Gonçalves et al., 2012).

The complement system is one of the first lines of defense against pathogenic infection by alerting hosts to the presence of potential pathogens as well as clearing pathogens. Complement can be activated by three different but partially overlapping routes: the classical pathway, the alternative pathway and the lectin pathway (Wang et al., 2008). All the three pathways merge at a

common amplification step involving C3, a central complement component that is part of all the three pathways, and proceed through a terminal pathway or lytic pathway that leads to the formation of a membrane attack complex which can directly lyse microbial cells (Wang et al., 2008; Brown et al., 2002; Holland and Lambris, 2002; Boshra et al., 2006; Fujita, 2002). The expression of Complement component 3b (C3b) was induced by immersion in the bacterial suspension and that induction was ~60% blocked by the presence of ammonia in viscera.

Defensins are a group of small antimicrobial peptides (AMP), which are a first line of host defence against invading pathogens (Zou et al., 2007), whereas intestinal alkaline phosphatase (Iap), a brush-border enzyme, has the ability to dephosphorylate and detoxify lipopolysaccharide and prevent bacterial invasion across the gut mucosal barrier (Bates et al., 2007). In this study the expressions of both were not affected significantly in any of the experiments.

The chronic exposure to HEA resulted in an inhibition of *saa* bacterial induction in gill for the injection challenge, although the induction of *saa* mRNA expression was only 6 fold when in viscera it was around 150 fold. There were no statistical differences for the other genes analysed by real-time qPCR for these experiments. It would thus appear that acclimation to HEA and/or the lower dose use generally do not lead to an impairment of the APR.

It is thus quite clear that HEA levels lead to an innate immunosuppression in zebrafish, and I think that the elevated whole-body cortisol levels after ammonia exposure may have been instrumental in that suppression of the fish immune system.

Conclusions

This study demonstrates that both acute and chronic ammonia exposure are capable of inhibiting the induction of some APPs, which are an important part of the acute phase response. This provides a clear link between HEA levels and disease susceptibility, and it is of particular pertinence to maintenance of fish in aquaculture and to monitor environmental health of fish in the wild.

CHAPTER 4

ENVIRONMENTAL AMMONIA-MEDIATED SUPPRESSION OF IMMUNE RESPONSE INVOLVES CORTISOL SIGNALING IN ZEBRAFISH

Abstract

It is known that high environmental ammonia (HEA) levels are related to increased vulnerability to different parasitic, viral and bacterial diseases in fishes, but the mechanisms involved are far from clear. We tested the hypothesis that elevated cortisol levels associated with HEA exposure suppress the lipopolysaccharides (LPS)-mediated innate immune response in zebrafish (*Danio rerio*). To this end, we tested the effect of exogenous cortisol administration and the role of glucocorticoid receptor (GR) signaling, using RU-486 (mifepristone), a glucocorticoid receptor antagonist, on the LPS-induced immune response to acute high environmental ammonia exposure in zebrafish. A panel of important immune-related genes, including suppressor of cytokine signaling (SOCS) 1, SOCS 2, SOCS 3, serum amyloid A (SAA), leukocyte cell-derived chemotaxin-2 (LECT2), haptoglobin (HP), hepcidin (HAMP), interleukin 1-β (IL1 β), and complement component 3b (C3b), were evaluated by real-time quantitative PCR. The results demonstrate that the elevated cortisol levels in response to environmental ammonia may be a key player in immune modulation, including upregulation of *socs* and downregulation of *saa*, *lect2* and *il1β*. Most of the HEA effect on immune response was attenuated by mifepristone underscoring a key role for GR signaling in the immune modulation. The upregulation of *socs* 1 and 2 by ammonia-mediated cortisol elevation may be an adaptive response that restricts activation of cytokine signaling. This would divert energy substrates away from endogenous (within the liver) immune-related pathways to those essential for coping with stress, including gluconeogenesis. LPS exposure suppresses the cortisol stimulated increases in *socs1* and *socs2* levels and downregulates *socs3* suggesting interaction with the cortisol signaling pathway. This study has revealed that ammonia's immune suppression, at least in part, is mediated through an increase in cortisol levels in zebrafish. We hypothesize that cortisol elevation ultimately increases the disease susceptibility associated with HEA levels in fishes.

Introduction

Ammonia is an unique toxicant, in that it is toxic to animals and at the same time produced by the animals as a result of protein catabolism. Although fish are more tolerant to high internal ammonia concentrations than terrestrial vertebrates (Wright, 1995), they are vulnerable to unusually high concentrations of environmental ammonia that occur naturally, or through sewage effluent discharges and agricultural run-off. In aquaculture, high levels can be problematic during rearing stages in which fish are held at high densities, or when using recirculating water systems, that may lead to accumulation of high ammonia levels. At toxic internal levels, ammonia is detrimental to central nervous system processes, oxidative metabolism, and may impair oxygen delivery (Wilkie, 1997). In extreme cases, ammonia toxicity, characterized by hyperactivity, convulsions, and coma, leads to death (Randal and Tsui, 1997).

Ammonia is also related to increased vulnerability to different parasitic, viral and bacterial diseases (Ackerman et al., 2006; Carballo et al., 1995; Evans et al., 2005; Liu, 2004). In a previous study, we demonstrated that both acutely and chronically elevated environmental ammonia levels were capable of suppressing the induction of genes of the acute phase response in zebrafish. We also observed elevated cortisol levels in response to high environmental ammonia (HEA) levels, that was proposed as a mechanistic explanation for the increased disease susceptibility that is associated with environmental ammonia exposure (Gonçalves et al., 2012).

Cortisol is a key modulator of physiological processes, including the stress response, metabolism, growth and immune response (Mommsen et al., 1999). In teleosts, cortisol is the primary circulating glucocorticoid and is released from the interrenal tissue (analogous to the adrenal cortex), distributed in the head kidney region and it is elevated during stress following activation of the hypothalamus-pituitary-interrenal (HPI) axis (Aluru and Vijayan, 2009). Mifepristone is a glucocorticoid receptor (GR) antagonist and has been used in a number of fish studies to reveal glucocorticoid signaling by blocking the effects of exogenously added cortisol (Mommsen et al., 1999).

The acute phase response (APR) is a set of immediate host inflammatory reactions, including remarkable changes in the concentrations of many plasma

proteins, known as the acute phase proteins (APPs; Gabay and Kushner, 1999), that protects the animal from tissue injury, infection and trauma (Uhlar and Whitehead, 1999). APR has been extensively studied in human and mouse, while some APPs were also isolated from some fish species (Bayne and Gerwick, 2001). Cytokines are an integral component of the adaptive and innate immune responses. Signal transduction via cytokine receptors is regulated by several mechanisms that control initiation, magnitude and duration of signaling pathways. Cytokine-induced suppressor of cytokine signaling (SOCS) family acts as feedback inhibitors of cytokine receptor activation by inhibiting a variety of signal transduction pathways (Alexander and Hilton, 2004; Jin et al., 2007).

For the current study, we have used zebrafish (*Danio rerio*). In recent years, zebrafish has become one of the most prominent vertebrate model organisms used to study the genetics underlying development, toxicology, immunity, and disease (Kari et al., 2007; Lin et al., 2006; van der Sar et al., 2004; Trede et al., 2004). We stimulated the innate immune response in zebrafish by injection with *Escherichia coli* lipopolysaccharides (LPS). LPS are also termed endotoxins, and are a cell wall component found in most Gram-negative bacteria. It has been shown in many studies, that LPS can induce the innate immune response in fishes (Gonçalves et al., 2012; Huttenhuis et al., 2006; Watzke et al., 2007).

The goal of the present study was to determine if cortisol mediates the environmental ammonia associated suppression of the innate immune response to LPS in zebrafish. To this end, two experimental studies were performed. In the first experiment, the effect of exogenous cortisol administration on the LPS-induced innate immune reponse was assessed in zebrafish. In the second experiment, the effects of glucocorticoid receptor blockade with mifepristone on APP induction and *socs* expression were assessed in zebrafish exposed to LPS either in the presence or absence of high environmental ammonia.

Material and Methods

Animals

Adult *Danio rerio* with a mass between 0.3-0.8 g were obtained from a local aquarium fish supplier and held at 26ºC in a 100L aquarium with aerated Porto city tap water (Na^+ 0.5 mM, alkalinity 50 mg/l $CaCO_3$, pH 8). The fish were fed with commercial fish food (TetraMin, Tetra, Germany) four times per day on automatic feeders (Eheim 3538, Germany) and were reared on a 12h light/dark cycle.

Experiment 1 – Cortisol and LPS

Zebrafish were anaesthetized (1:10000 tricaine methanesulfonate (MS222), Alpharma, UK, pH 7.5, adjusted with $NaHCO_3$), weighed and intraperitoneally injected with 50 µg cortisol /g wet mass in a coconut oil implant. An injection volume of 5µl/g was used. Another group of fish was injected with coconut oil (vehicle alone) and served as the control. After 48h, fish were anaesthetized as described above, and intraperitoneally injected with 10µg/g LPS (Sigma Aldrich, St. Louis MO USA) (infected groups) or PBS (sham groups), in an injection volume of 10µl/g. After 24h all fish were sampled (n=7, see section 2.4).

Experiment 2 – Mifepristone, LPS and acute HEA

Zebrafish were anaesthetized (1:10,000 MS222), weighed and 50% of the fish were intraperitoneally injected with saline (0.9% w/v; groups I, II, III, IV; table 4.1) and served as controls. The other 50% were injected with 100 µg/g mifepristone (Sigma Aldrich) diluted in 0.5% ethanol. An injection volume of 5µl/g was used (Groups V, VI, VII, VIII). After 48h, fish were again anaesthetized as described above, and intraperitoneally injected with PBS (sham groups: I, II, V, VI) or 10µg/g LPS (infected groups: III, IV, VII, VIII) in an injection volume of 10µl/g. The zebrafish were allowed to recover in tanks in the absence (groups I, III, V, VII) or presence of 1mM ammonia (NH_4Cl; groups II, IV, VI, VIII) at pH 8.0 buffered with 10mM Tris-HCl. After 24h all fish were sampled (n=8; see section 2.4).

Table 4.1: Summary of experiment 2 treatment groups.

TANK	I	II	III	IV	V	VI	VII	VIII
Mifepristone	-	-	-	-	+	+	+	+
LPS	-	-	+	+	-	-	+	+
HEA	-	+	-	+	-	+	-	+

Sampling

Zebrafish were heavily anaesthetized (1:5000 MS222), weighed and standard length measured. Fish were then killed by decapitation on ice, with a transverse cut anterior to the pectoral fins. Viscera (that includes liver, intestine, pancreas and spleen) were excised and kept in RNAlater (Sigma-Aldrich) and the remaining carcasses were snap frozen in liquid nitrogen and stored at -80˚C for later analysis.

Total RNA Extraction and cDNA synthesis

Total RNA was isolated using commercial silica based columns (Aurum Total RNA Mini Kit, Bio-Rad, Hercules, CA, USA). All steps were performed according to the manufacturer's instructions, including the on-column treatment of isolated RNA with RNase-free DNase I. Total RNA was quantified at 260/280nm using a Nanodrop spectrophotometer (Wilmington, DE) and integrity confirmed by electrophoresis (1.2% formaldehyde agarose gel; Mini-Sub Cell GT Cell, Bio-Rad) and stored at -80ºC until further use. The cDNA was synthesized from 1µg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol.

Primers

Primers were designed from gene sequences available in Ensembl (www.ensembl.org) and GenBank (http://www.ncbi.nlm.nih.gov/), or taken directly from published studies (Table 4.2). Primers were designed using Primer3 (Rozen and Skaletsky, 2000).

Table 4.2: Primer pairs (sense and anti-sense, respectively) for real time qPCR with original GenBank accession number. Primers with an asterisk (*) were designed with Primer3 and reference sources are given for the remainder.

Gene Name	GenBank Accession No.	Forward and reverse primer sequences (5'-3')	Ref
ef1α	NM_131263	TGGGTGTTGGACAAACTGAA CAACACCACCAGCAACAATC	*
saa	NM_001005599.1	CGGGGTCCTGGGGGCTATTG GTTGGGGTCTCCGCCGTTTC	Lin et al., 2007
lect2	BC162786.1	TTCTACTTTTGGCTGTGCTA ACATCCTCTTTTTTGGTTAC	Lin et al., 2007
hp	XM_689364.3	TGATGCTACAGCCTCTACGG GTGTTCTGGAAGCCTGGATG	Lin et al., 2007
hamp	NM_205583.1	CACAGCCGTTCCCTTCATAC TCAGATGTTGGTTCTCCTGC	Lin et al., 2007
Il1β	AY340959	TGGACTTCGCAGCACAAAATG GTTCACTTCACGCTCTTGGATG	Van der Sar et al 03
tfa	NM_001015057	TGCAGAAAAAGCTGGTGATG ACAGCATGAACTGGCACTTG	*
c3b	NM_131243	CAGTGGGAATATGTTGGCATTG TTAGCTGCCCTTCATAACCTGTT	*Rojo et al., 2007*
socs1	AJ810174.1	AGTCGTCAGAGCGACGTTTT CCGTCGCATCTTTCCATAAT	*
socs2	NM_001114550.1	ACCTGCGGATCGAATACAAG TAATGGTCCACGAGATGCAC	*
socs3	NM_213304.1	GCACCTTCCTGGTGCGGGAC ACTGTCCCGGCTGAGGGCAT	*
gr	EF567112.1	ACAGCTTCTTCCAGCCTCAG CCGGTGTTCTCCTGTTTGAT	*

Real-time quantitative RT-PCR (qPCR)

Relative levels of mRNAs for innate immunity genes were quantified by qPCR analysis using SYBR green on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad). Each cDNA sample was diluted and then 5 µl added to a reaction mix containing 10 µl of 2× iQ SYBR Green Supermix (Bio-Rad), and 250 nM of each primer in a total reaction volume of 20 µl. Each sample was prepared in duplicate for each gene. The cycling profile was the following: 94 ºC for 3.5 min, 40 cycles of 94 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 30 s. A melting curve was generated for every PCR product to confirm the specificity of the assays and a dilution series was prepared to check the efficiency of the reactions. *Ef1*α was used as the housekeeping gene. The comparative CT method ($2^{-\Delta\Delta CT}$ method) based on cycle threshold (CT) values was used to analyze the expression levels of the genes of interest.

Whole-body cortisol extraction and measurement

Whole zebrafish were homogenized using a bead mill in 1ml dH_2O (6800 RPM 2x 30s Precellys24). Homogenates were centrifuged for 2 min at 14000 RCF at room temperature (Eppendorf MiniSpin Plus, Germany), and the supernatant was decanted and extracted with 5 ml of diethyl ether, dried and resuspended in 100µl of extraction buffer. Cortisol in the whole-body extracts of individual fish was measured using a Cortisol Elisa Kit (Neogen, USA) and all steps were performed according to the manufacturer's instructions following validation for use with zebrafish. Cortisol values were corrected for dilution steps and expressed as ng cortisol per g of fish wet mass.

Ammonia measurements

Water ammonia levels were measured using an enzymatic microplate technique, modified from Bergmeyer and Beutler (1985). Water samples were added in triplicate to a 96-well microplate on ice. Ammonia standards (0 to 350 µM) were also added in triplicate on each microplate. Reagent mixture (2 mM NADH, 11 mM 2-Oxoglutarate, 0.56 mM ADP, 155 mM TEA, final concentrations in assay mixture) was added to each well, and thoroughly mix for 2 min. Absorbance was read at 340 nm before and after adding 1.48 U GDH (Bio-Tek PowerWave 340 microplate reader, Vermont, USA). A linear regression was performed with the standards (ammonia concentration versus difference in absorbance at 340 nm), and ammonia concentration of each sample was

calculated through a linear equation.

Statistics

The data are presented as means ± standard error of the mean (SEM). Sigma Stat (3.0 SPSS, Chicago IL USA) was used for all statistical analyses and $P<0.05$ was considered significant. One-way and two-way analysis of variance followed by Student-Newman-Keuls post-hoc tests were used to compare differences.

Results

Cortisol and LPS

Cortisol treatment significantly elevated *socs1a* (14-fold), *socs2* (12-fold), but not *socs3* mRNA levels in zebrafish (Fig. 4.1a-c). Cortisol induced up-regulation of *socs1a* and *socs2* was inhibited by the presence of LPS (~70%; Fig 4.1a and 4.1b, respectively). *socs3* mRNA expression was significantly reduced in the LPS treated groups in comparison with the groups injected with PBS (sham; Fig. 4.1c).

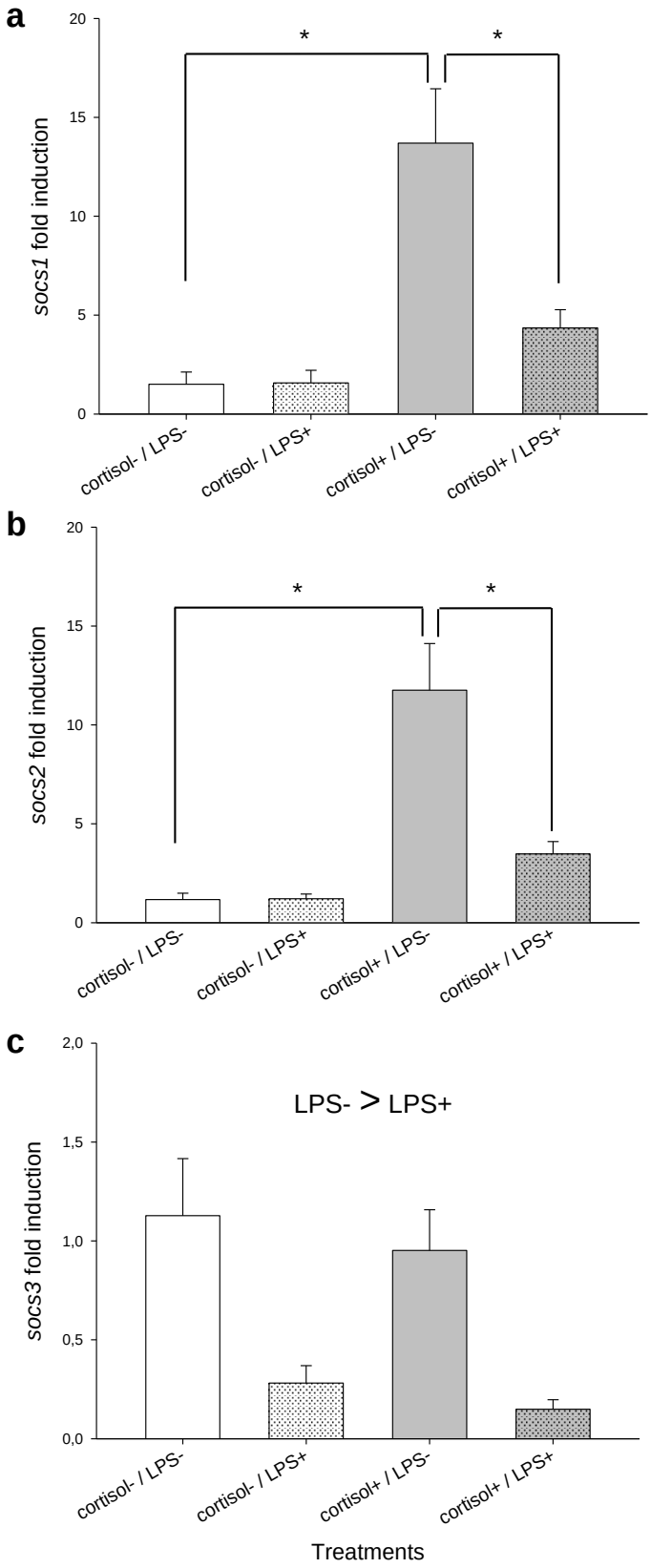

Figure 4.1. Effect of cortisol and LPS treatment on mRNA expression of *socs* genes. Effect of cortisol on (a) *socs*1, (b) *socs*2 and (c) *socs*3 expression in viscera of zebrafish in the presence and absence of LPS. Zebrafish were intraperitoneally injected with

cortisol (10 mg/ml) diluted in coconut oil (grey bars), an injection volume of 5µl/g was used. Another group of fish was injected with coconut oil (vehicle alone) and served as the control. After 48h, fish were intraperitoneally injected with 10µg/g LPS (hatched bars) or PBS, an injection of 10µl/g was used. Gene expression was measured by q-PCR relative to *ef1*α. Data analyzed by 2-way ANOVA and post hoc Student–Newman–Keuls test. All values represent means ± SE (n = 7), bars with different letters are significantly different.

LPS treatment significantly elevated *saa* (125-fold) and *lect2* (142-fold) mRNA expression (Fig. 4.2a and 4.2b). Cortisol treatment had no effect on basal expression levels of these genes; however, it significantly suppressed the LPS induced up-regulation by more than 70%.

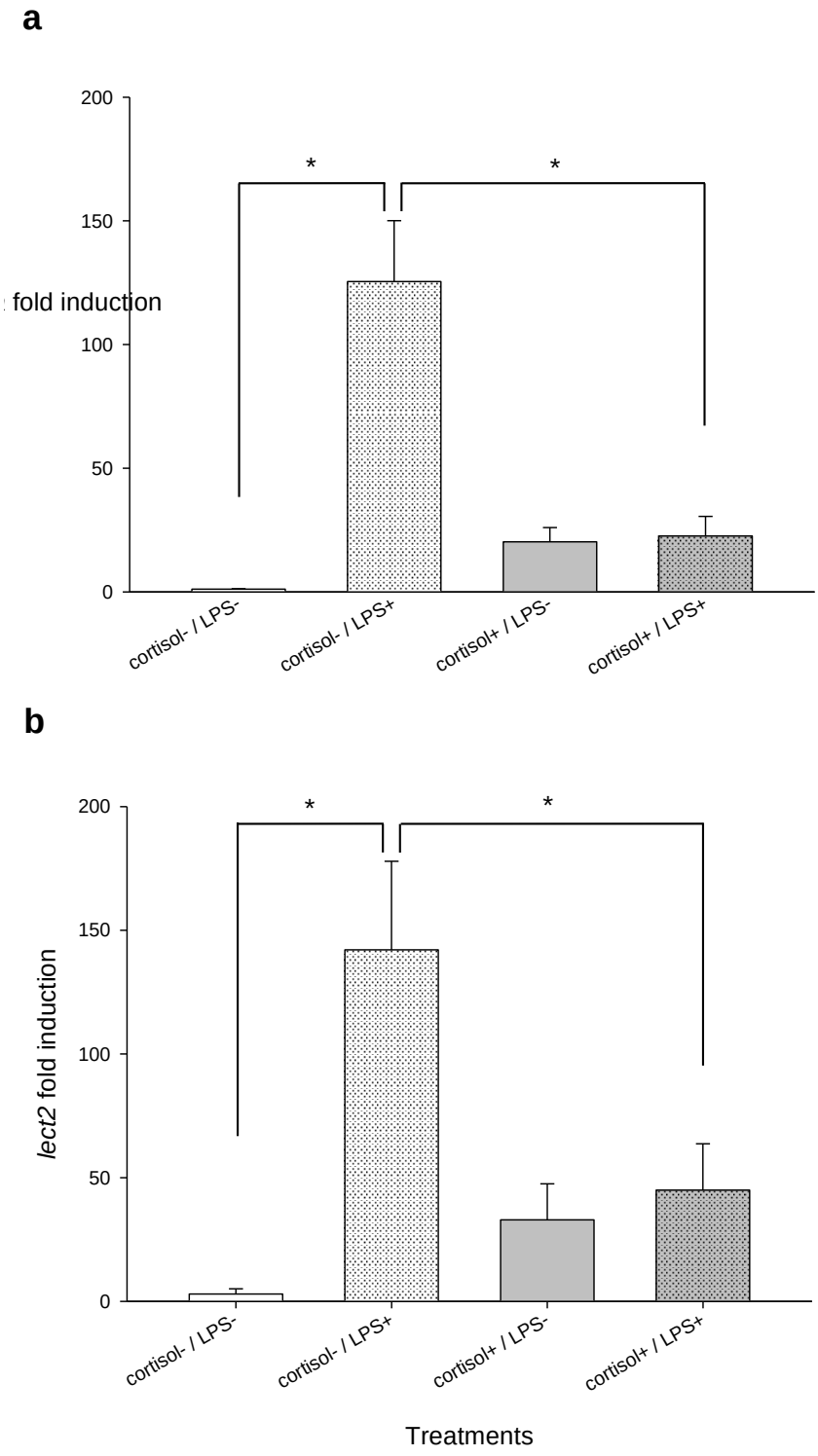

Figure 4.2. Effect of cortisol and LPS treatment on mRNA expression of acute phase proteins. Effect of cortisol on (a) *saa* and (b) *lect2* gene expression in viscera of zebrafish in the presence and absence of LPS. Zebrafish were intraperitoneally injected with cortisol (10 mg/ml) diluted in coconut oil (grey bars), an injection volume of 5µl/g was used. Another group of fish was injected with coconut oil (vehicle alone) and served as the control. After 48h, fish were intraperitoneally injected with 10µg/g LPS (hatched

bars) or PBS, an injection of 10µl/g was used. Gene expression was measured by q-PCR relative to *ef1*α. Data analyzed by 2-way ANOVA and post hoc Student–Newman–Keuls test. All values represent means ± SE (n = 7), bars with different letters are significantly different.

Whole-body cortisol levels were significantly higher in the groups treated with cortisol in comparison with the groups treated with coconut oil (vehicle alone). LPS treatment did not significantly alter cortisol levels (Fig. 4.3).

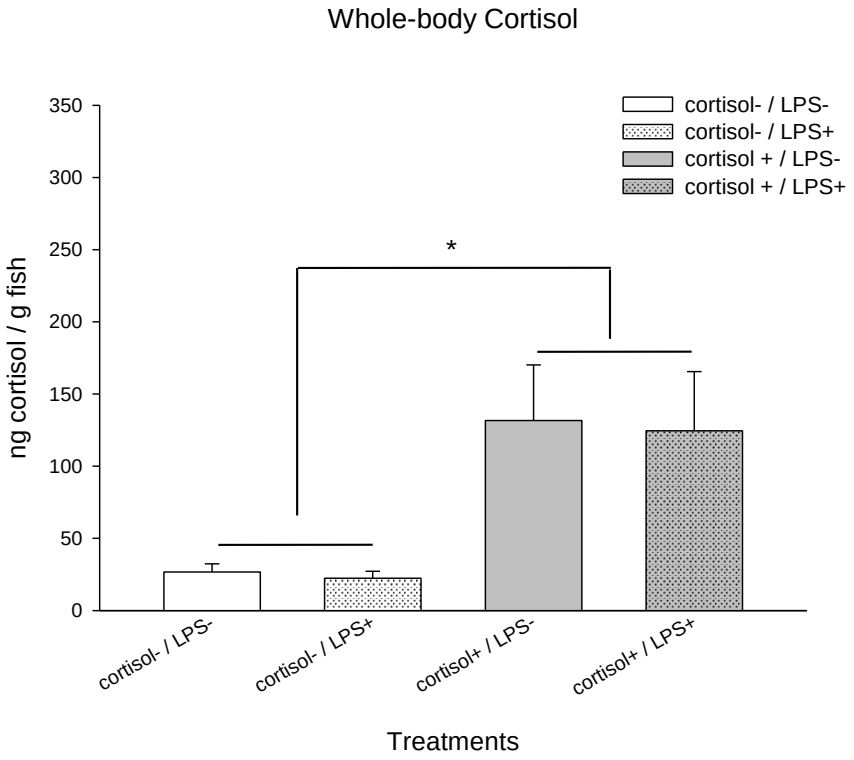

Figure 4.3. Whole-body cortisol (ng/g fish) levels of zebrafish. Effect of cortisol in the presence and absence of LPS. Zebrafish were intraperitoneally injected with cortisol (10 mg/ml) diluted in coconut oil (grey bars), an injection volume of 5µl/g was used. Another group of fish was injected with coconut oil (vehicle alone) and served as the control. After 48h, fish were intraperitoneally injected with 10µg/g LPS (hatched bars) or PBS, an injection of 10µl/g was used. Data analyzed by 2-way ANOVA and post hoc Student–Newman–Keuls test. All values represent means ± SE (n = 7). Asterisk (*) indicate differences between cortisol groups and groups injected with vehicle alone.

Mifepristone, LPS and acute HEA

The *socs1* mRNA expression increased significantly with acute high environmental ammonia (HEA) exposure (15-fold) and the HEA induced up-regulation was impaired by LPS by 50%, for the groups not treated with mifepristone. On the other hand, the treatment with mifepristone blocked that ammonia induction of *socs1* mRNA, and the subsequent LPS attenuating effect (Fig. 4.4). *socs2*, *socs3* and *GR* mRNA expression showed no significant changes (data not shown).

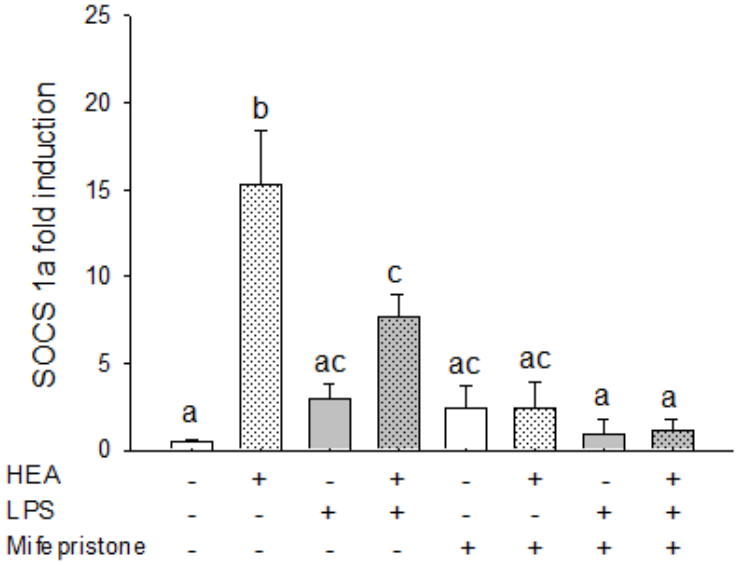

Figure 4.4. Effect of ammonia and LPS on *socs1* mRNA expression, in the presence and absence of mifepristone. Effect of ammonia and LPS on *socs1* mRNA transcript level in viscera of zebrafish, in the presence and absence of mifepristone. Zebrafish were intraperitoneally injected with 100 µg/g mifepristone, diluted in ethanol, an injection volume of 5µl/g was used. Other fish were injected with saline (0.9% w/v) and served as the control. After 48h, fish were intraperitoneally injected with 10µg/g LPS (grey bars) or PBS, an injection of 10µl/g was used. They were allowed to recover in tanks in the absence or presence of 1mM ammonia (hatched bars) at pH 8.0. Data analyzed by 1-way ANOVA and post hoc Student–Newman–Keuls test. All values represent means ± SE (n = 7). Bars with different letters are significantly different.

The expression of a panel of genes for innate immune response induction in zebrafish was also evaluated: serum amyloid A (SAA), leukocyte cell-derived chemotaxin 2 (LECT2), haptoglobin (Hp), hepcidin (HAMP),

interleukin 1β (IL1β), complement component 3b (C3b) and transferrin (TFA). *saa*, *lect2*, *hp*, *hamp*, *Il1β* and *c3b* transcript levels increased significantly with LPS treatment (6-200 fold; Fig. 4.5a-f) in the groups without. Although it seems that HEA impairs that LPS induction for all genes, there is only a significant effect for *il1β* (Fig. 4.5e). For the groups treated with, the impairment caused by the ammonia exposure appears to be completely absent. Once again these results seem show a trend but are not significant for all genes due to the high variability between animals (Fig. 4.5a-f). *tfa* transcript levels did not show any significant changes (data not shown).

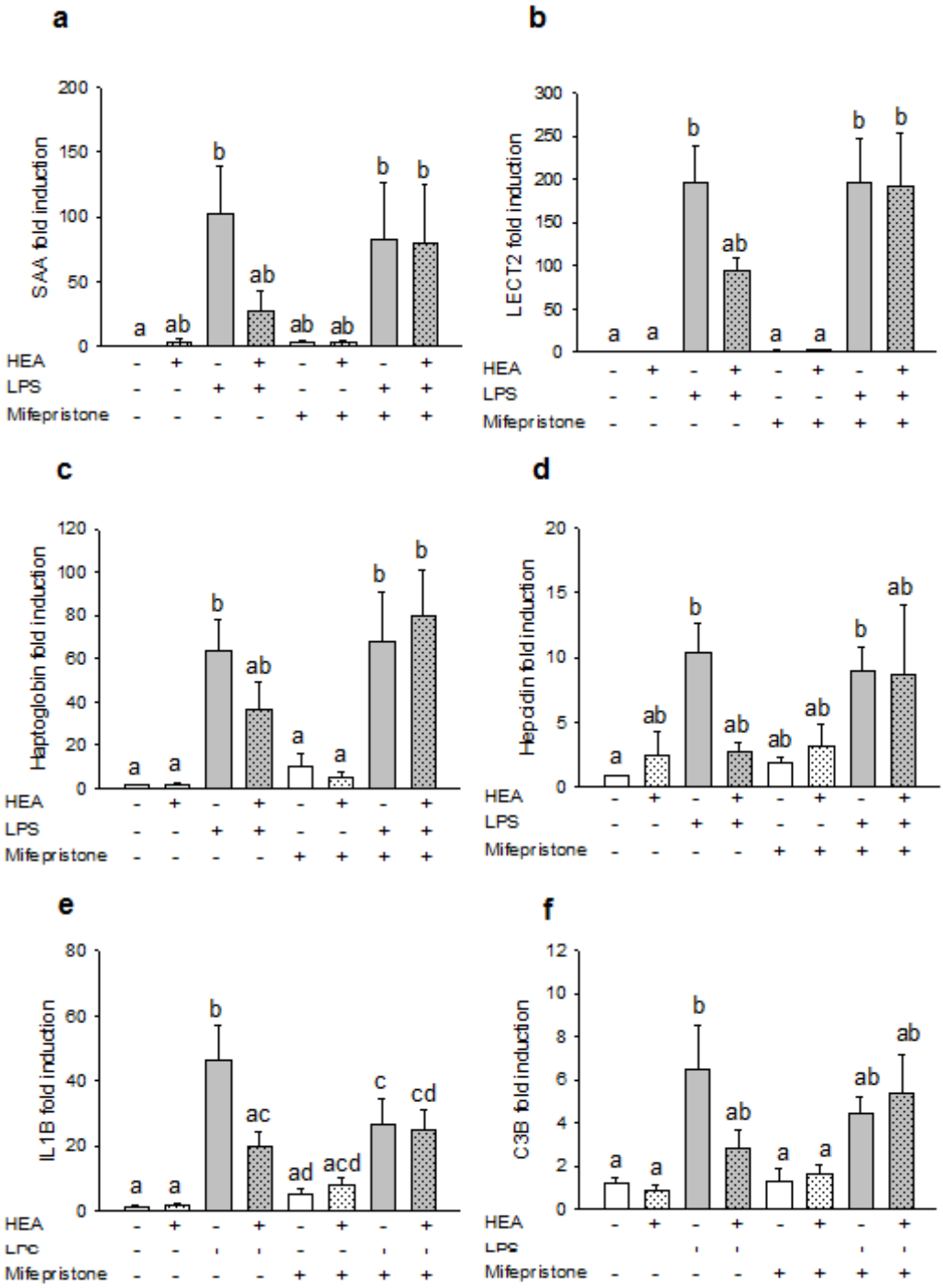

Figure 4.5. Effect of ammonia and LPS on mRNA expression of immune response genes, in the presence and absence of mifepristone. Effect of ammonia and LPS on (a) *saa*, (b) *lect2*, (c) *haptoglobin*, (d) *hepcidi*n, (e) *il1β* and (f) *c3b* gene expression, in viscera of zebrafish, in the presence and absence of mifepristone. Zebrafish were intraperitoneally injected with 100 µg/g mifepristone, diluted in ethanol, an injection volume of 5µl/g was used. Other fish were injected with saline (0.9% w/v) and served as the control. After 48h, fish were intraperitoneally injected with 10µg/g LPS (grey bars) or PBS, an injection of 10µl/g was used. They were allowed to recover in tanks in the absence or

presence of 1mM ammonia (hatched bars) at pH 8.0. Data analyzed by 1-way ANOVA and post hoc Student-Newman-Keuls test. All values represent means ± SE (n = 7). Bars with different letters are significantly different.

Whole-body cortisol levels were significantly higher in the groups exposed to acute ammonia levels, in the absence of, and LPS treatment had no effect. In the presence of mifepristone, the HEA increase in whole-body levels of cortisol was blocked completely (Fig. 4.6).

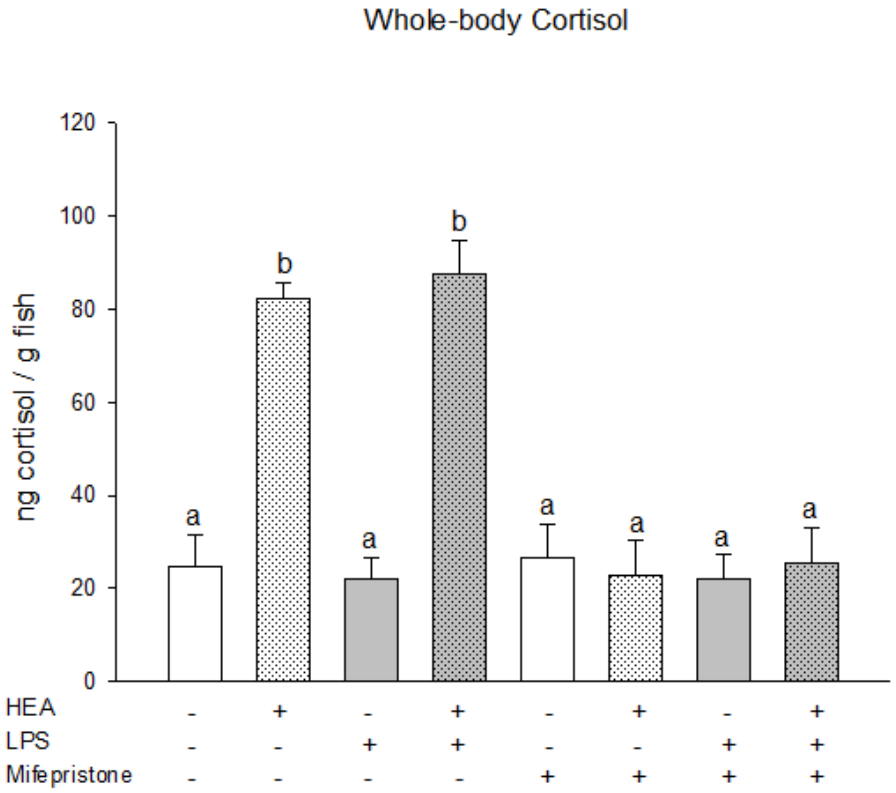

Figure 4.6. Whole-body cortisol (ng/g fish) levels of zebrafish. Effect of ammonia and LPS, in the presence and absence of mifepristone. Zebrafish were intraperitoneally injected with 100 µg/g mifepristone diluted in ethanol, an injection volume of 5µl/g was used. Other fish were injected with saline (0.9% w/v) and served as the control. After 48h, fish were intraperitoneally injected with 10µg/g LPS (grey bars) or PBS, an injection of 10µl/g was used. They were allowed to recover in tanks in the absence or presence of 1mM ammonia (hatched bars) at pH 8.0. Data analyzed by 1-way ANOVA and post hoc Student-Newman-Keuls test. All values represent means ± SE (n = 8). Bars with different letters are significantly different.

Discussion

This study demonstrates that elevated cortisol levels through exogenous administration and in response to environmental ammonia modulate mRNA levels of genes encoding key mediators of innate immunity in zebrafish. Suppressors of cytokine signaling (*socs*) mRNA levels were upregulated by exogenous cortisol and HEA; whole-body cortisol levels were elevated by HEA exposure but not LPS in zebrafish. It was also demonstrated that mifepristone, a well established GR antagonist, tends to mask the HEA/cortisol effect on innate immunity-related genes suggesting a role for GR signaling in this process. Given the similar direct effects of HEA/cortisol, we can infer that the ammonia effect on immunosuppression seen in our previous study (Gonçalves et al., 2012; Chapter 2) is partly mediated by cortisol, the principal corticosteriod in teleost fishes (Mommsen et al., 1999).

The acute phase response (APR) is the immediate set of host inflammatory reactions that counteract challenges such as tissue injury, infection and trauma (Uhlar and Whitehead, 1999). At the site of invasion by a micro-organism and the place of tissue injury, a number of responses of the tissue itself are initiated. Pro-inflammatory cytokines are released, and the vascular system and inflammatory cells are activated (Gruys et al., 2005). During the APR there are also remarkable changes in the concentrations of many plasma proteins, known as the acute phase proteins (APPs) (Gabay and Kushner, 1999). An acute-phase protein has been defined as one whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 25 percent during inflammatory disorders (Morley and Kushner, 1982). Various acute phase proteins were isolated from rainbow trout (Bayne and Gerwick, 2001). Our results showed that cortisol supressed the LPS-induced acute phase proteins *saa* and *lect2's* mRNA expression levels. Environmental ammonia impaired the LPS-induced up-regulation of *il1β*, and we can see the same trend for all other gene transcipts, although significant differences are lacking. These results are in agreement with previous studies demonstrating that stress-induced glucocorticoid levels downregulate cytokine mRNA levels in gilthead seabream (*Sparus aurata*) head kidney cells (Castillo et al., 2009). Also, work done in rainbow trout

(*Oncorhynchus mykiss*) macrophage cell line showed that high glucocorticoid levels down-regulate cytokine mRNA levels (Castro et al., 2011). Cortisol also modulated the LPS stimulated gene expression of *tnfα2*, *il8* and *saa* in trout hepatocytes (Philip et al., 2012). Specifically, the suppression of LPS-induced expression by cortisol supports an immunosuppressive effect of this steroid on fish immune cells (Stolte et al., 2008; MacKenzie et al., 2006; Castro et al., 2011). Engelsma and colleagues showed that cortisol was able to inhibit *in vitro* constitutive expression of *il1β* transcripts, but that the addition of cortisol simultaneously with LPS could not susbstantially inhibit *il1β* mRNA expression in common carp (*Cyprinus carpio)* (Engelsma et al., 2000). Furthermore, mifepristone seemed to attenuate the effects of cortisol/HEA on the immune-related genes, although the effects were not statistical different because of the high variability between individual zebrafish. These observations demonstrate an important role for glucocorticoid receptor signaling in regulating immune response during stress; although, the failure of mifepristone to completely block the ammonia/cortisol effect suggests that other non-GR mediated signaling may also be involved in zebrafish (Aluru and Vijayan, 2007). Cytokines are low molecular weight cell derived glycoproteins, controlling the cell to cell communication on a variety of target cells responsible for an immune response. They are important for mounting immune responses and regulating host defense network. These molecules regulate local and systemic immune inflammatory and regulatory events. Cytokines include interleukins, tumor necrosis factors, interferons, colony stimulating factors and chemokines (Krebs and Hilton, 2001). An important regulator of the inflammatory response to cytokine stimulation are the SOCS proteins (Kile and Alexander, 2001). SOCS family members modulate signaling by several mechanisms, which include inactivation of the Janus kinases (JAKs), blocking access of the signal transducers and activators of transcription (STATs) to receptor binding sites, and ubiquitination of signaling proteins and their subsequent targeting to the proteasome. SOCS proteins, therefore, form part of a classical negative feedback circuit (Krebs and Hilton, 2001). Multiple isoforms of *socs* genes, including *socs1, socs2* and *socs3*, have been cloned and sequenced in trout (Wang et al., 2008; Wang et al., 2010); however, it remains to be established whether the SOCS functions in fish are similar to that described in mammals (Kile and Alexander, 2001). In this study, exogenous cortisol upregulated *socs1* and *socs2*, and furthermore that upregulation was inhibited by LPS injection.

socs3 was not affected by cortisol but was reduced by LPS. Our results reveal, for the first time, the upregulation of *socs1* mRNA levels by acute environmental ammonia in zebrafish, and that LPS inhibits that up-regulation by 50%. mifepristone, a well established GR antagonist (Aluru and Vijayan, 2007), attenuated the cortisol increase in response to HEA and effects seen with HEA on the LPS-induced APR. This can be explained by the ability of mifepristone to reduce the steroidogenic capacity of interrenal tissue (Alderman et al., 2012), thus lowering the whole-body cortisol in the HEA group . These results are in line with work done by Philip and colleagues (2012) that showed that cortisol modulated the molecular immune response in trout hepatocytes. In their study cortisol increased mRNA abundances of *socs1* and *socs2*, and these cortisol-mediated effects were rescued by mifepristone. Furthermore, bioinformatic analysis revealed putative glucocorticoid response elements on the promoter of the SOCS1 gene in zebrafish (Philip unpublished) supporting GR regulation of this gene. Croker and colleagues (2008) showed that upregulation of SOCS inhibited JAK/STAT signaling, which play an important role in suppressing cytokine signaling and modulating other cellular energy demanding pathways. For instance, growth hormone (GH), which is a key regulator of postnatal somatic growth, signals primarily through the JAK2–STAT5b pathway. It has been shown that SOCS proteins interact with GH receptor (GHR) signaling, and overexpression of SOCS2 interferes with the JAK2–STAT5b pathway (Croker et al., 2008). Also, phenotypically SOCS2-deficient mice resemble GH-transgenic mice, displaying increased body weight resulting from enhanced bone size and an enlargement of most organs, supporting a negative regulation of GH actions by SOCS genes (Croker et al., 2008). Taking these results together, we hyphothesize that upregulation of *socs* gene expression by ammonia/cortisol may be an adaptive response that limits infection-induced inflammatory responses and the associated metabolic costs by restricting activation of cytokine signaling. As GH shares a common pathway with SOCS, this would also divert energy resources away from growth promoting action of GH during stress in fish. Consequently, energy substrates may be reallocated away from the inflammatory response and growth to pathways essential for coping with stress.

There are various studies demonstrationg that LPS challenge stimulates *socs*1, *socs*2 and *socs3* expression in fish immune cells (Jin et al., 2007a,b, 2008). However, in this study LPS downregulated the cortisol induced *socs1* and *socs2* expression, while expression of *socs*3 was reduced in the groups treated

with LPS in comparison with the groups with PBS. Philip and colleagues (2012) showed no changes with LPS in *socs*1 and *socs*2 expression, while mRNA abundance of *socs*3 was reduced. It is possible that the expression may be transient as recently seen in yellow perch (*Perca fluescens*) liver where *socs*1 and *socs*3 expression peaked between 6 and 12 h and was back to the pre-LPS exposure level at 24 h (Shepherd et al., 2012). Overall, the temporal changes and the precise role of SOCS proteins in response to an endotoxin shock in fish remains enigmatic.

Together, these results suggest that the stress hormone cortisol and the elevated cortisol levels in response to environmental ammonia may be the driver in an important regulation of key innate immunity genes. This would confirm the hypothesis that the impact of environmental ammonia on immune suppression, at least in part, is mediated through an increase in cortisol levels in zebrafish, providing a mechanistic link between increased disease susceptibility and HEA levels in fishes. We hypothesize that this cortisol action may have adaptive significance by diverting energy resources away from the inflammatory response and growth, and allowing the animal to metabolically cope with the enhanced energy demand associated with stress.

Conclusions

This study demonstrated that exogenous cortisol and elevated cortisol levels in response to environmental ammonia modulates mRNA transcript levels of genes encoding key mediators of innate immune response in zebrafish. It remains to be determined if high levels of cortisol in fish are the only mechanism for immune-supression observed when fish are exposed to high levels of environmental ammonia, and further work needs to be done. It is of particular pertinence to understand the link between HEA levels and disease susceptibility, to monitor environmental health of fish in the wild and for maintaining fish in aquaculture.

CHAPTER 5

GENERAL DISCUSSION

Introductory Remarks

Over the last decades, there have been various studies that have shown that exposure to high environmental ammonia (HEA) levels increased susceptibility to different parasitic, bacterial and viral diseases in fishes (Carballo et al., 1995; Carballo and Munõz, 1991; Evans et al., 2005; Hanson and Grizzle, 1985; Liu, 2004). However, the mechanism of action of ammonia has remained largely unknown.

The original aim of the present thesis was to provide new insights into the complexity of the relationship between high environmental ammonia levels and disease susceptibility in fishes, not only by testing the hypothesis that ammonia acts through immunosuppression of the innate immune response, but also by elucidating if cortisol mediates that suppression. The observations and results from the past years and their contribution to an increased knowledge of the interaction between HEA and increased vulnerability to disease in fish will be discussed in the following sections.

Does ammonia act through immunosuppression of the APR?

In the initial experiments of Chapter 2, a number of APPs that responded to LPS were identified for subsequent experiments with HEA exposure. These included SAA, LECT2, HAMP and Hp. These APPs are well documented in various fish species (Boshra et al., 2006; Holland and Lambris, 2002; Lin et al., 2007; Rodrigues et al., 2006; Vilarroel et al., 2008). The function of most APPs has not been totally elucidated. However, APPs are regarded as having general functions in opsonization and trapping of microorganisms and their products, activating complement, binding cellular remnants like nuclear fractions, neutralizing enzymes, scavenging free haemoglobin and radicals, and modulating the host's immune response (Gruys et al., 2005). The *saa* induction that was observed in zebrafish is in line with previous studies of challenges with LPS done in Atlantic salmon (Jorgensen et al., 2000) and rainbow trout (Villarroel et al., 2008). The *lect2* induction by LPS observed in this study correlates with the work of Lin and colleagues (2007) that showed *lect2* induction upon infection of zebrafish with *Aeromonas salmonicida* and *Staphylococcus aureus*. The observation of *hp* induction with LPS is in agreement with work done in zebrafish after infection with *A. salmonicida* and *S. aureus* (Lin et al., 2007). *hp* mRNA induction has also been observed in rainbow trout after exposure to *Vibrio anguillarum*, by microarray analysis (Gerwick et al., 2007). Also, *hamp* gene expression increased with LPS, and this observation is consistent with studies of bacterial infection in sea bass (Rodrigues et al., 2006), zebrafish (Lin et al., 2007), and catfish (Bao et al., 2005). And to test the role of pro-inflammatory cytokines in zebrafish APR, mRNA levels of *Il1β* were measured after exposure to LPS in a time course series. The observed *Il1β* induction suggested that pro-inflammatory cytokines may play an early, pivotal role in induction of APPs in fish, similar to the mechanism described in mammals (Lin et al., 2007), The *Il1β* elevated response observed also correlates with experiments conducted previously with LPS or bacterial exposures in zebrafish (Novoa et al., 2009; Pressley et al., 2005; Lin et al., 2007). These results demonstrated that APPs in zebrafish may play an essential role in anti-infection as in other species. The second part of Chapter 2

took a closer look at the LPS-HEA effects. In the acute experiment the presence of HEA blocked the LPS induction of *saa*, *hp* and *hamp* gene expression by approximately more than 50%. In the chronic study, HEA affected the LPS induction of *lect2* by almost 50%. As hypothesized, it was quite clear that ammonia exposure lead to an innate immunosuppression in these zebrafish.

In Chapter 3 the experimental work was extended to determine if acute (24h) or chronic (14 days) exposure to HEA levels also affected the APR to bacterial infection. The induction of a number of genes of the APR was examined in viscera or gill of zebrafish, after an infection challenge by immersion to *Edwarsiella tarda*, to mimic natural infection conditions in fish, or by intraperitoneal injection which allowed for more controlled challenge conditions. Gene expression analysis by real-time qPCR revealed that the presence of HEA had an immunosuppressive effect on the induction of a number of APPs that respond to infection, such as *hamp*, *lect2*, *saa*, *hp* and *c3b*, in the immersion and/or injection bacterial challenges. The inhibition of APP gene expression was present in viscera and in gill, although the fold induction to bacterial challenge was higher in the former. This likely happened because the changes in the concentrations of APP are due largely to changes in their production by hepatocytes (Russel et al., 2006). Furthermore, it was observed that after i.p. injection with four different doses of *E. tarda* (10^6, 5 x 10^6, 10^7 and 5 x 10^7 CFU), zebrafish mortality was higher in the presence of HEA at 24 h, 48 h and 72 h post infection. The *E. tarda* LC50 value for 72 h was three times lower for fish exposed to ammonia versus control fish. Elevated ammonia concentrations have previously been shown to damage gills in various fish species (Lang et al., 1987; Carballo and Muñoz, 1991; Plumb, 1984), and this structural gill pathology has been linked to functional consequences in salmonids (Woodward et al., 1983). It is well known that the gill epithelium is critical to overall fish health as it is the primary site of O_2 uptake, ammonia excretion and ion regulation (Lease et al., 2003). HEA also interferes with mucus renewal by retarding mucus production by the mucous cells (Lang et al., 1987) and reducing the presence of defensive substances in the mucus (Mock and Peters, 1990). Several functional roles have been attributed to mucus in fish, including an important role in disease resistance (Shephard, 1994). These two factors together may facilitate bacterial colonization, which can be translated into higher fish mortality when in the presence of ammonia. These results support the prediction that ammonia increases susceptibility to different

bacterial and viral fish diseases (Carballo et al., 1995; Carballo and Muñoz, 1991; Hanson and Grizzle, 1985; Plumb, 1984).

Is cortisol involved in the HEA-mediated suppression of the APR?

It is known that the primary response of fish to stress is the production of catecholamine and cortisol by chromaffin cells and interrenal tissue (Harris and Bird, 2000) and there are studies showing that fishes exposed to ammonia have significantly higher plasma cortisol levels (Ackerman et al., 2006; Carballo et al., 1995; Tomasso et al., 1981). Also, Ramsay and colleagues (Ramsay et al., 2006, 2009) showed that whole-body cortisol levels of zebrafish are an indicator of crowding stress and acute net handling stress. Varsamos and colleagues (2006) showed that temperature stress during early life stages of seabass (*Dicentrarchus labrax*) increased plasma cortisol concentrations and susceptibility to nodavirus. In Chapter 2 was demonstrated that both acute and chronically elevated ammonia levels increased whole body cortisol levels. In contrast to other studies using acute stressors, where elevated whole body cortisol levels were rapidly brought back to pre-stress levels (Ramsay et al., 2006; Fuzzen et al., 2010), continuous HEA resulted in a persistent elevation of cortisol levels. It has been suggested that cortisol may act to inhibit IL-like factors (Engelsma et al., 2002) and have suppressive effects on both specific and non-specific components of the immune system (Fevolden et al., 1993; Harris and Bird, 2000). In fish, increases in cortisol have been demonstrated to attenuate the production of pro-inflammatory cytokines (Castro et al., 2011; Engelsma et al., 2002; Zou et al., 2000).

In Chapter 4 of the present thesis it was demonstrated that exogenous cortisol exposure up-regulated the suppressors of cytokine signaling *socs1* and *socs2*, and furthermore that up-regulation was inhibited by LPS. *socs3* was not affected by cortisol. Also, exogenous cortisol down-regulated the LPS-induced acute phase proteins *saa* and *lect2's* mRNA expression levels. Acute environmental ammonia exposure, which increased fish cortisol levels, also up-regulated *socs1* mRNA levels and inhibited the LPS-induced up-regulation of *il1β*. The same trend is present for other APP genes analyzed, although statistical significance is lacking. Given the similar direct effects of HEA and cortisol, it is possible to infer that the ammonia effect on immunosuppression showed in the previous chapters of this thesis is mediated by cortisol, the principal corticosteriod in teleost fishes (Mommsen et al., 1999). These results

are in agreement with the work done by Engelsma and colleagues (2000) who demonstrated that cortisol was able to inhibit *in vitro* constitutive expression of *il1β* transcripts in *Cyprinus carpio*. Also, studies done in fish cell lines have shown that high glucocorticoid levels down-regulate cytokine mRNA levels (Castro et al., 2011; Philip et al., 2012). Furthermore, mifepristone, a well established GR antagonist (Aluru and Vijayan, 2007), attenuated the cortisol response and brought down most of the effects seen with HEA by inhibiting the hypothalamic-pituitary-interrenal axis functioning and hence the lower whole-body cortisol in the HEA groups with mifepristone; although not all effects were statistical different because of the high variability between individual zebrafish. These results are in line with work done by Philip and colleagues (2012) that showed that cortisol modulated the molecular immune response in trout hepatocytes. In their study cortisol increased mRNA abundances of *socs1* and *socs2*, and these cortisol-mediated effects were rescued by mifepristone. This is also in agreement with the attenuated plasma cortisol response to an acute stressor seen with mifepristone treatment in rainbow trout (Alderman et al., 2012). Furthermore, bioinformatic analysis revealed putative glucocorticoid response elements on the promoter of the *socs* 1 gene in zebrafish (Philip unpublished) supporting GR regulation of this gene. Croker and colleagues (2008) showed that upregulation of *socs* inhibited JAK/STAT signaling, which plays an important role in suppressing cytokine signaling and modulating other cellular energy demanding pathways. For instance, growth hormone (GH), which is a key regulator of postnatal somatic growth, signals primarily through the JAK2–STAT5b pathway. It has been shown that SOCS proteins interact with GH receptor (GHR) signaling, and over expression of SOCS2 interferes with the JAK2–STAT5b pathway (Croker et al., 2008). Also, phenotypically SOCS2-deficient mice resemble GH-transgenic mice, displaying increased body weight resulting from enhanced bone size and an enlargement of most organs, supporting a negative regulation of GH actions by *socs* genes (Croker et al., 2008). Taking these results together, it may be possible that the up-regulation of *socs* gene expression by ammonia/cortisol is an adaptive response that limits infection-induced inflammatory responses and the associated metabolic costs by restricting activation of cytokine signaling, and diverting energy resources away from growth promoting action of GH during stress in fish, which would reallocate energy substrates to pathways essential for coping with stress. This would confirm the hypothesis that environmental ammonia acts, at least in part,

through an increase in cortisol levels in zebrafish, that provokes an immune-suppression, and ultimately increases the disease susceptibility associated with HEA levels in fishes.

Concluding Remarks and Future Perspectives

Elevated cortisol levels delivered exogenously or elevated in response to high environmental ammonia (HEA) levels modulate mRNA transcript levels of genes encoding key mediators of innate immune response in zebrafish. With this thesis a greater understanding of the relationship between increased disease susceptibility in fish in response to HEA exposure has been achieved, but there is much work to be done and many questions remain unanswered.

1. It remains to be determined if high levels of cortisol in fish are the only cause for the immunosuppression observed when fish are exposed to HEA. Additional studies are clearly warranted.

2. Since the gill is exposed to the external environment, it is an important frontline defense which is also affected by HEA. An APR to bacterial challenge has been demonstrated in fish gill; however, additional responsive immune genes likely come into play. A transcriptomic (next generation sequencing) approach could be taken to expand our understanding of the role of the gill in the innate immune response.

3. Zebrafish are an important model organism; however, other more appropriate species of ecological and/or aquacultural relevance should also be investigated to extend these findings.

It is of particular importance to understand the link between HEA levels and disease susceptibility, in order to monitor environmental health of fish in the wild and for maintaining fish in aquaculture.

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